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# *The Electrical Charge Characteristics of Muscle Proteins*

*Thesis submitted for the degree of  
Doctor of Philosophy  
in the Discipline of Biophysics*

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## ABSTRACT

In this study, muscle proteins were extracted, and concentrated gels of these proteins were produced. Microelectrode techniques were developed in order to measure the electrical charge in these gels.

Results of the charge measurements confirm that myosin and actin, respectively, are the principle contributors of the charge found in the A- and I-bands of intact muscle fibres. Results also generally accord with the charge predicted by the amino-acid composition of these proteins. However, the charge on myosin in particular is lower than that found in the A-band.

Myosin charge was measured under a variety of experimental conditions. Only in combination with C-protein did the charge approach that found in intact muscle.

Gels of F-actin were measured at both high and low concentrations. The low-concentration gel (23-75 mg/ml) was found to change charge upon stirring. Further work is indicated to elucidate this phenomenon.

The results of this study suggest that variations in charge may be related principally to two factors. First, conformational changes in the protein molecule are likely to affect ion binding. Second, different experimental conditions and protein combinations are likely to affect the degree of order of the protein gel.

## ACKNOWLEDGEMENTS

I would like to pay tribute to all the people who have helped ensure the completion of this work:

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## CHAPTER 1

### INTRODUCTION

1.1 The process of muscle contraction, and the roles played by the constituent proteins, continue to challenge and fascinate. The present study was designed to contribute to this quest by examining the charge characteristics of the two major muscle proteins, myosin and actin.

1.2 Striated muscle consists of groups of muscle fibres, which in turn are made up of bundles of myofibrils. The composition of each myofibril includes the contractile proteins myosin and actin, a number of structural or other proteins including C-protein, and the regulatory proteins tropomyosin and the three troponins C, I and T. Within the myofibril, myosin and actin contribute, respectively, to the thick and thin filaments, arranged in a highly ordered lattice which is the basis of the repeat unit, the sarcomere (Fig 1.1).

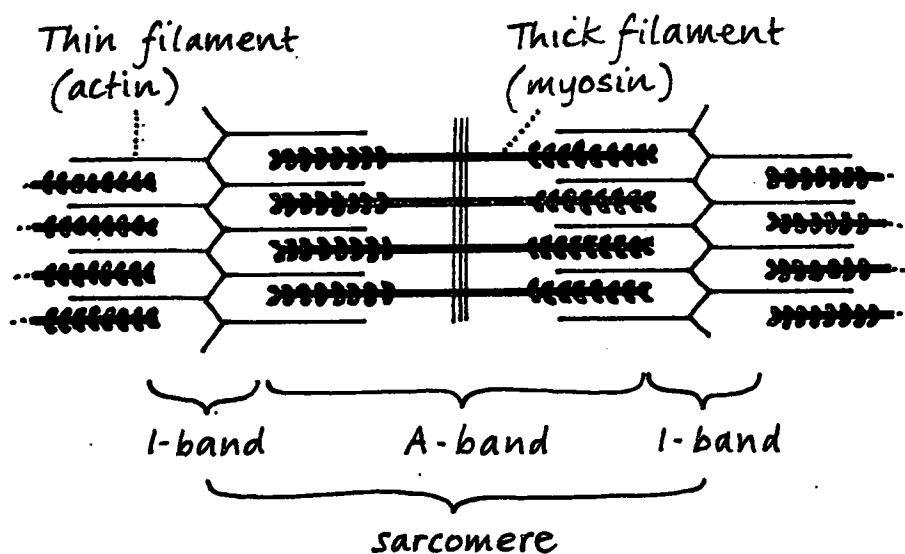


Fig 1.1: Myofibril filament lattice, in simplified diagrammatic form

1.3 In the generally-accepted sliding filament hypothesis of muscle contraction (Huxley & Niedergerke 1954, Huxley & Hanson 1954) the degree of overlap of the filaments becomes greater or less as the sarcomere contracts or stretches.

1.4 Microelectrode studies of intact muscle fibres (glycerinated or chemically skinned) were pioneered by Collins and Edwards in the early 1970s (Collins & Edwards 1971). Their approach made use of the fact that intact muscle is a fixed polyelectrolyte system with a measurable electrical potential, the Donnan potential (Chapter 5, section 5.1).

1.5 These and subsequent studies show a negative fixed charge in the myofibril, which varies according to different conditions such as the state of contraction or the ionic strength of the bathing solution (eg Elliott 1973; Naylor et al 1985). More recently it has proved possible to insert a microelectrode into a specific part of the sarcomere, and separate measurements of charge on the thick and thin filaments have been made (Bartels & Elliott 1981, 1985). To complement these observations, the present work was undertaken to develop a method of direct measurement of the charge on the main constituents of the filaments, the isolated proteins myosin and actin.

1.6 A myosin molecule has two globular heads with an average diameter of about 90 angstroms, and a flexible tail about 1500 angstroms long (Slayter & Lowey 1967); it has a molecular weight of about 478,000 daltons (Harrington 1979) (Values given here may be lower than the most recent estimates). The action of papain splits this molecule into a tail section called myosin rod, with a molecular weight of about 220,000 daltons, and two head sections, called subfragment 1 or S-1, each with a molecular weight of about 115,000 daltons. The action of trypsin splits the molecule

at a different site, the hinge region part way down the tail, and results in two fragments, heavy meromyosin (HMM) including the two heads, and light meromyosin (LMM) (Fig 1.2). In this study only the products of papain digestion were examined.

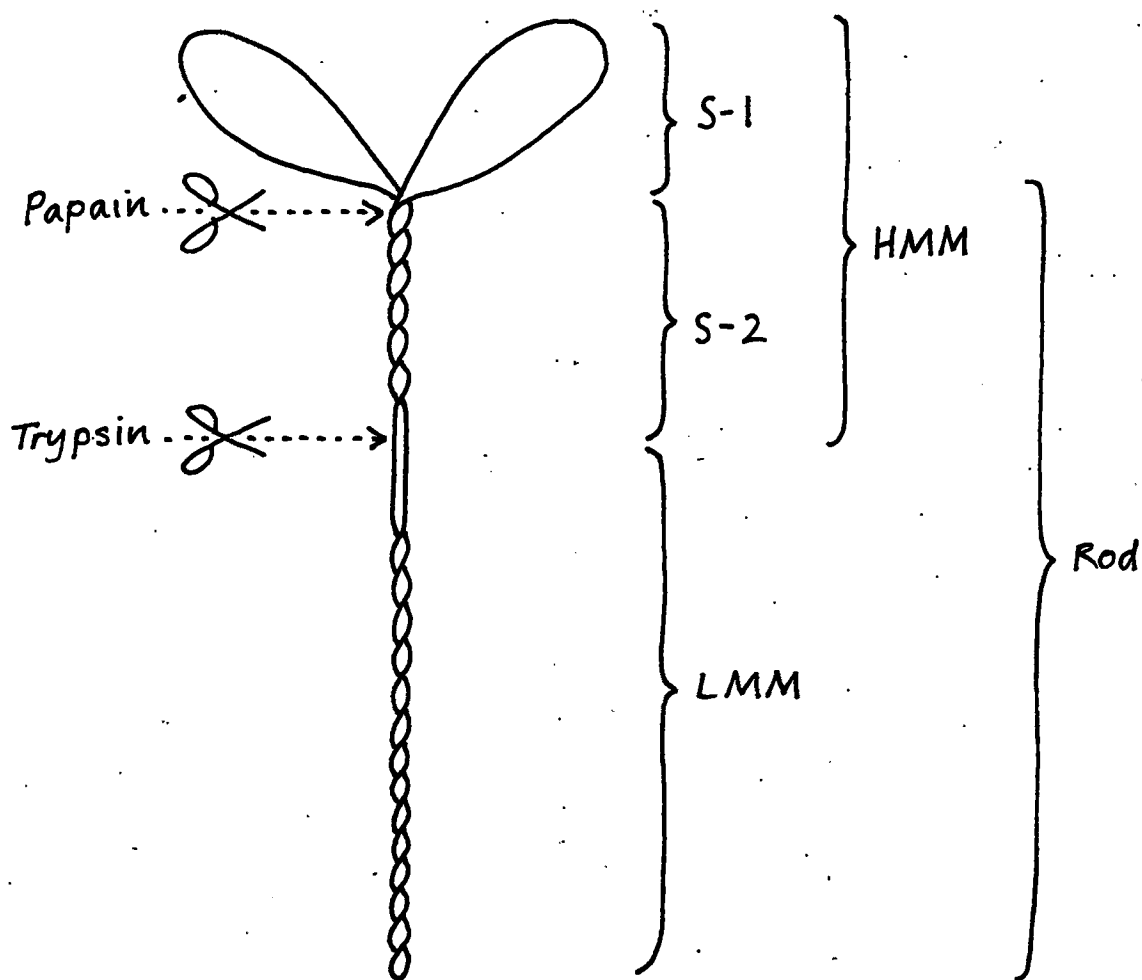


Fig 1.2: Schematic diagram of the myosin molecule, showing fragmentation products

1.7 Actin, the principal constituent of the thin filament, is a polypeptide chain in a globular conformation (G-actin) with a volume of about 45,000 cubic angstroms. It associates into long strands or filaments (F-actin) in physiological salt conditions (Asakura et al 1963). Amino acid sequencing (Collins & Elzinga 1975) gives a molecular weight for G-actin of 41,785 daltons.

1.8 In the thin filament, actin is complexed with the regulatory proteins tropomyosin and the troponins C, I and T. Tropomyosin is a two-stranded coiled coil of alpha-helices with a molecular weight of 65-70,000 daltons. Troponin consists of three polypeptide chain sub-units: troponin C, 17,965 daltons; troponin I, 21,897 daltons; and troponin T, 30,500 daltons. Tropomyosin forms a complex with F-actin in solution, and the troponin complex binds strongly to tropomyosin (but not directly to actin). The model structure for the thin filament complex (Fig 1.3) was proposed by Ebashi et al in 1969 and slightly modified in 1972 (Ebashi 1972).

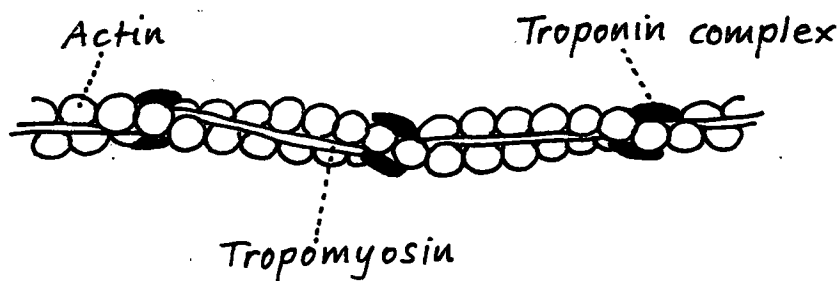


Fig 1.3: Model of the actin-tropomyosin-troponin complex of the thin filament, after Ebashi 1972

1.9 C-protein is a single polypeptide chain with a molecular weight of 140,000 daltons, which binds strongly to myosin and to myosin rod and LMM (Moos et al 1975). It is thought to have a regulatory role, perhaps in myosin filament assembly.

1.10 There is a rapidly-growing body of biochemical and structural information about these proteins, but comparatively little is known about their electrical charge characteristics. Muscle proteins are polyelectrolytes: they are macromolecules which in solution have an electrical charge, and in combination form a highly-charged polyelectrolyte gel.

1.11 The net charge arises from the charged groups, both positive and negative, in the composition of the protein molecule. The acidic groups, which at physiological pH (6.5-7.0) result in a negative charge, are aspartate (pK 3.86) and glutamate (pK 4.25). The basic groups, resulting in a positive charge, are lysine (pK 10.53) and arginine (pK 12.48); histidine (pK 6.0) is also basic, but most histidine groups are likely not to be ionised at physiological pH.

1.12 Most biological macromolecules, including muscle proteins, have more negative than positive groups in their composition: they thus carry a net negative charge. Because this charge is produced by ionised groups it is pH-dependent, becoming more negative if the pH is increased and less negative if it is decreased, until the isoelectric point of the protein is reached. At physiological pH the surface potential on a muscle filament is in the order of a few tens of millivolts, a magnitude which is comparable with the membrane potential of the cell.

1.13 These charges have important implications for the interactions between macromolecules. In a salt solution, cations (positive ions), will be attracted to the



negative surfaces of the molecules, and anions (negative ions) will be repelled. This results in a gradation of ions in the solution around each molecule, forming an electrical double layer. This in turn gives rise to a contour of electrical charge, a potential well, between adjacent molecules and between adjacent proteins. In this situation the concentration of the salt solution will determine the strength of the repulsive force generated between the molecules: the lower the concentration, the higher will be the repulsive force.

1.14 It has long been known (eg Rome 1967) that changes in electrical conditions, such as those brought about by changes to pH or ionic strength, can cause the whole muscle lattice to swell or shrink. Millman and Nickel (1980) studied repulsive pressure systems, including tobacco mosaic virus (TMV) and vertebrate striated muscle, in order to model the long-range electrostatic forces operating in such systems. Elliott (1992) uses the calculation of the electric fields around cylindrical protein filaments to examine the potential well between myosin filaments and between myosin and actin filaments, raising the possibility of an ion-pumping function for the myosin head.

1.15 However, these studies of the operation of electrical forces in muscle are isolated examples, and all rely on indirect measurements of charge characteristics, or on calculations of charge derived from other observed parameters. The fact that muscle protein charge characteristics have been relatively neglected, by comparison with muscle protein structure and biochemistry, is likely to be largely due to the difficulty of making direct measurements of protein charge under given conditions.

1.16 Elliott et al (1985) sum up the position thus: "A direct physical approach to any working mechanism of microscopic dimensions should include (1) the

distribution of matter, and importantly (2) the distribution of electric charge. Some understanding of these two distributions would provide an essential step in defining a working mechanism in physical terms. While the experimental paradigm in the biological sciences usually includes substantial effort to define the distribution of matter in systems which may have been simplified, in solution for example, the distribution of electric charge is often overlooked or ignored in kinetic schemes, largely because of the technical difficulties in obtaining a reliable index of charge patterns at high spatial and temporal resolution. Even in contracting muscle, where finite changes in the distribution of contractile material occur on a measureable time scale, very little effort has been made to analyse the mechanism in the physical terms of charge density and distribution".

1.17 However, all these proteins have one property which makes direct measurement of their charge possible: they will cross-link to form stable arrays. They are thus capable of forming gels, either alone or in combination.

1.18 The aim of this work, therefore, was to exploit this property by producing protein gels of a reliable consistency and appropriate concentration, in a form which would allow their repeated measurement with a microelectrode. This enables the Donnan potential of the gel (section 5.1.1) to be measured, and the fixed internal charge (2.6.1-3) calculated. The objective was to develop the techniques of gel production and microelectrode measurement, while making an initial series of measurements on these proteins under a range of conditions, in order to delineate a field for further exploration.

## CHAPTER 2

### METHODS

#### Section 2.1 : Protein Extraction

2.1.1 Myosin and actin were extracted from skeletal and psoas muscle of rabbit, using a method derived from that described in Offer et al (1973).

The rabbit was freshly killed, and back and leg muscles were immediately dissected out in a cold-room, bringing its temperature down rapidly to about 4°C. The muscle tissue was put through a domestic mincer (a hand mincer was found to be more reliable than an electric one), and weighed after mincing. It was then mixed with three volumes of chilled Guba-Straub solution (0.3M KCl, 0.15M  $\text{KPO}_4$ , pH 6.5) and stirred with an electric paddle stirrer for ten minutes, in ice.

After rapid addition of chilled water to a volume of sixteen times muscle weight, the tissue was allowed to strain slowly through cheesecloth, then squeezed. Straining typically took 1-3 hours. The muscle residue was reserved for subsequent actin extraction (see below), and the filtrate containing the myosin was diluted, slowly, two- to three- fold with chilled water, and left in the cold-room to settle, normally overnight.

2.1.2 Myosin was prepared from the resulting sediment. When this had settled to less than about 1.5 litres, the supernatant was drawn off by syphoning, and the residue was centrifuged in 300ml bottles in a Pegasus ultracentrifuge at 10K rpm for ten minutes, at 3° C. The resulting pellets were resuspended in buffer consisting of 0.3M KCl, 1mM EDTA, 10mM  $\text{KPO}_4$ , pH 7.0, in ice, and dispersed with a Jencons

hand homogenizer. The suspension was recentrifuged at 12K for 30 minutes at 3° C. Any traces of fat were then sucked off by pipette, and the solution was decanted. Five volumes of chilled water were added to the solution, and it was centrifuged at 10K for ten minutes. (To handle this volume, five centrifuge runs were usually necessary at this stage.) The supernatant was discarded. Following these two precipitation stages, the crude myosin was clarified by suspending the pellets in a solution of 1M KCl, 3mM EDTA, pH 7, and adding 1M K-P<sub>i</sub> buffer\*, pH 7.0, and water, to give a final concentration of 50mM K-P<sub>i</sub> and 0.5M KCl in the final volume. Careful stirring with a small spatula and a glass rod ensured complete dispersal of the pellet. This suspension was then centrifuged at 30K for ninety minutes. The solution was decanted, and the residue discarded.

This solution was mixed with an equal volume of 70% saturated ammonium sulphate solution, in ice (35% ammonium sulphate fractionation), and centrifuged for ten minutes at 10K. Solid ammonium sulphate was added to the supernatant at the rate of 31g per litre (40% fractionation), stirred in ice, and centrifuged as before. The pellets were then washed by re-suspending in 42% saturated ammonium sulphate solution, and finally centrifuged at 10K for ten minutes. (This procedure usually took 2-3 days). The resulting myosin pellets could be stored in the refrigerator or cold-room for an indefinite period; it was found (by SDS gel electrophoresis and by charge measurement) that myosin stored in this way showed no signs of deterioration after several months.

---

\* Phosphate buffer used in these experiments was KPO<sub>4</sub>, pH 7.0. Stock solution was made from 1M KH<sub>2</sub>PO<sub>4</sub> titrated to pH 7.0 with KOH 5N.

In order to prepare myosin for ultracentrifuging into the small, concentrated gels in which Donnan potential measurements were made, a small quantity (about 10ml) of the ammonium sulphate precipitate (myosin pellet) was placed in a dialysis bag, and dialysed overnight against a minimum of two changes of 0.5M KCl in order to remove all traces of ammonium sulphate. The myosin solution was then re-dialysed, again overnight, in at least two changes of one of the standard experimental solutions (see Table 2.1). Since myosin is soluble only in solutions containing more than about 0.4M salt, this procedure caused its re-precipitation. The protein concentration was then measured with a spectrophotometer, in a 0.1mm sandwich cuvette, using an extinction co-efficient of  $0.52 \text{ cm}^2/\text{mg}$ . Typically at this stage the concentration was about 35mg/ml.

2.1.3 Actin preparation used the residue from the initial protein extraction stage. This residue was in some cases stored, frozen, for a period of up to six months before further processing; in other cases the actin preparation took place immediately following the extraction stage. No differences between the final actin gels were observed; however, there appeared to be a slightly higher actin yield from the frozen residue. After thawing (where necessary) the muscle residue was allowed to rest at room temperature for two hours. It was then suspended in five volumes of 0.4%  $\text{NaHCO}_3$ , 0.0001M  $\text{CaCl}_2$ , and stirred mechanically, with a paddle stirrer, for thirty minutes at room temperature. This suspension was strained through cheesecloth. The liquid was discarded, and the residue re-suspended in one volume of chilled 0.01M  $\text{NaHCO}_3$ , 0.01M  $\text{Na}_2\text{CO}_3$ , 0.001M  $\text{CaCl}_2$ , in ice. After stirring for ten minutes, it was diluted in 10 volumes (typically about five litres) of water at room temperature, and strained again in cheesecloth, squeezing out surplus liquid by hand.

	KCl, mM	MgCl <sub>2</sub> , mM	K(P), mM*
1.5 A	150	7.5	30
A	100	5	20
0.75 A	75	3.75	15
0.5 A	50	2.5	10
0.2 A	20	1	4

TABLE 2.1 : Standard experimental solutions. All solutions

\* K(P) = phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>), pH 7.0.

This residue was twice washed in pure acetone: one litre of acetone was added, the suspension stirred for 30 minutes at room temperature, then strained residue was minced or electrically blended, using all-metal apparatus, to reduce it to fine, short fibres (1-3mm long); this took, typically, several hours, processing a very small quantity at a time, since actin fibres easily clog the apparatus. The minced actin was then re-washed in acetone to remove any final traces of fat; this was repeated several times until the acetone stayed clear. After straining once more, the actin was spread between clean filter paper overnight, to allow the acetone to evaporate completely. The actin was then carefully re-blended. The resulting actin powder was divided into 10g quantities and wrapped in aluminium foil, labelled and dated. It could then be stored in the freezer indefinitely, although if left unfrozen it would quickly deteriorate.

Actin was extracted from this powder only when needed, as the resulting preparation remains viable for only two to three days.

Extraction was begun by suspending 10g of actin powder in 200ml ice-cold water, stirring for 20 minutes. The preparation was then centrifuged at 18K for 20 minutes, and the supernatant filtered off through glass wool, discarding the pellets. The supernatant was re-spun at 40K for 30 minutes, and decanted into a 250ml graduated cylinder. KCl was added to 50mM, and  $MgCl_2$  to 1mM, the solution mixed thoroughly by gently inverting the cylinder, and left to stand at room temperature for at least two hours, or overnight, to polymerize.

After the polymerization had been confirmed (either by checking the birefringence, or simply by observing the viscosity), the solution was transferred to a beaker in ice.

KCl was added to 0.8M, and TRIS buffer\* to 10mM, and the solution was stirred magnetically for 20 minutes. It was then centrifuged at 42K for ninety minutes, and the supernatant decanted and discarded. The pellets were re-suspended in a depolymerizing solution containing 10mM TRIS, 0.1mM  $\text{MgCl}_2$ , 0.2mM ATP, and thoroughly homogenized with a Jencons hand homogenizer, in ice. To this homogenate was added  $\text{MgCl}_2$  to 3mM, and after stirring for a few minutes it was centrifuged at 42K for ninety minutes at 3° C. The resulting actin pellets were then dialysed against the experimental solutions in the same way as was the myosin. Concentration of actin at this stage was typically 25-65 mg/ml.

Actin was used both at this concentration range and at the higher range of 150-250mg/ml, achieved by ultracentrifuging in 2ml tubes (see section 2.2 below).

2.1.4 Tropomyosin-troponin was extracted from acetone-ether dried actomyosin powder. This was prepared from actomyosin which had been extracted overnight at pH9 in high salt, followed by precipitation at low salt. 5g of actomyosin powder was suspended overnight in 100ml buffer containing 1M KCl, 2mM DTT\*\*. The suspension was then centrifuged at 30K for ninety minutes. The pellet was discarded and the supernatant was dialysed, again overnight, against 2mM DTT, 25mM MOPS, pH 7, followed by 40% and then 60% ammonium sulphate fractionations, discarding the pellets. The final yield was typically about 250mg tropomyosin-troponin, which was stored in a refrigerator before use.

---

\* TRIS used in these preparations was pH 7.5. Stock solution was made from 1M TRIZMA base titrated to pH 7.5 with HCl.

\*\* Di-thio-threitol (DTT) was included to prolong the life of the preparation.



2.1.5 S-1 (Myosin subfragment 1) was prepared from standard myosin precipitate. This was first dialysed overnight against 0.5M KCl, 1mM EDTA. It was then clarified by centrifuging at 30K for thirty minutes, and re-dialysed against 0.2M  $\text{NH}_4\text{COOCH}_3$ , 1mM EDTA. The resulting precipitate was digested with papain, using 0.02 mg/ml papain, 0.2mM EDTA, 0.025mM DTT, for 8 minutes 45 seconds (standard in Dr C Moos' laboratory) at room temperature. Immediately this time had elapsed the digestion was stopped by the addition of iodo-acetic acid (IAA) to 1mM, in ice. The pH was adjusted to 6.5. The solution was then centrifuged at 30K for an hour. The pellets were discarded and the supernatant was precipitated with 1.5 volumes of saturated ammonium sulphate. Finally, this was centrifuged at 8K for ten minutes. The resulting S-1 pellets were stored in a refrigerator and used within three days.

2.1.6 Myosin rod was prepared by the same method as S-1, with the papain digestion being allowed to proceed for twenty minutes. After the digestion had been stopped and the solution spun, the supernatant (containing the S-1 fragment) was discarded and the pellets dissolved in 2M KCl. This solution was diluted to 0.5M KCl, and three volumes of EtOH added. This was stirred for two hours at room temperature.

The resulting precipitate was suspended and dialysed overnight in 0.5M KCl, 10mM K-Pi. It was then centrifuged at 30K for an hour, and the pellets discarded. The supernatant myosin rod solution was re-precipitated by dilution with water at pH 6.5, and centrifuged at low speed. The pellets were then re-dissolved in 0.5M KCl and clarified by centrifuging at 30K for thirty minutes. The myosin rod was finally precipitated by dialysing in an experimental solution in the same way as myosin.

2.1.7 Phosphorylated myosin was prepared from myosin solution from which all ammonium sulphate had been removed. This solution was dialysed overnight in 25mM TRIS base, 12.5mM MgAc<sub>2</sub>, 0.1mM CaCl<sub>2</sub>, 1mM DTT, pH 7.8. The myosin was then subjected to reaction with 5mM ATP, 0.01mM MLCK (myosin light chain kinase)\*. This reaction was allowed to proceed for fifteen minutes at room temperature, stirring, and was then stopped with saturated ammonium sulphate. The myosin precipitate was dialysed against 0.5M KCl, and the resulting solution was re-precipitated by dialysis in an experimental solution. The phosphorylated myosin was used at once.

2.1.8 C-protein was prepared by Dr Carl Moos's laboratory at the State University of New York, Stony Brook, by column separation and purification (Offer et al, 1973). It was used ex stock.

2.1.9 SDS gels run on sample preparations of each protein confirmed the expected composition. It was not thought necessary to run SDS gels on each individual preparation.

2.1.10 The experimental solutions (Table 2.1) were used principally in order that data might be correlated with the results of work being done in the same laboratory on intact (glycerinated or chemically skinned) fibres, in which these solutions were standard (see, for example, Bartels & Elliott, 1985). Solution A was originally designed to approximate to physiological conditions.

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\* MLCK was stock held in Dr Moos' laboratory. Alternative phosphorylation procedures are now in use.

## **Section 2.2 : The Protein Gel**

2.2.1 Accurate calculation of protein charge depends upon the accuracy and consistency of measurements both of potential difference and of protein concentration. In order, therefore, to produce the final, highly-concentrated protein gel in a form in which these measurements could be made with the minimum disturbance to the structure of the gel, disposable centrifuge tubes were chosen: these had the advantage that they could be cut away leaving the gel intact (see Fig 2.1). Initially 10ml tubes were tried, but these proved both cumbersome and wasteful of protein. For the substantive experiments on high-concentration gels, therefore, 2ml tubes were used, first Beckman cellulose nitrate tubes, catalogue number 303369, size  $1\frac{5}{16}$ " x  $\frac{5}{16}$ " diameter, later succeeded by Beckman acetate tubes of identical dimensions.

2.2.2 Estimating the quantity of protein which would produce a gel large enough to measure adequately, yet small enough to be contained intact in the tube base after one side of the tube had been cut away (Fig 2.1), was initially a matter of trial-and-error. Experimentally, between 4 and 6mg of protein (whether myosin, actin, or a combination of proteins) was found to produce a satisfactory pellet.

2.2.3 Where a gel containing two or more proteins was required, the proteins were combined, in known and precise proportions, at this stage. In cases where one protein would not, on its own, cross-link to form a gel, but would bind to another, gelling, protein, the non-gelling protein was added in excess of the stoichiometric ratio, in the expectation that the two would bind stoichiometrically and that the non-bound protein (the excess) would be removed in the supernatant. This was checked spectrophotometrically.

2.2.4 Where variations from the standard A series of solutions were sought (eg absence of magnesium; addition of ATP) these too were introduced at this stage, as well as to the subsequent bathing solution. The protein was allowed to equilibrate in the new solution before ultracentrifuging.

2.2.5 In order to fill a 2ml tube (volume in practice was found to be about 2.1ml) a quantity of protein (between 6 and 12mg) was made up to 3ml with dialysate or a dilution thereof, to produce a series of different experimental ionic strengths (Table 2.1 above). The protein was measured precisely, by volume, with a glass syringe and fine-bore piping. Clean syringes were then used for repeated mixing, in ice, of each 3ml sample, in order to ensure complete and uniform dispersal of the protein. Disposable tubes, appropriately labelled, were filled completely, capped, and centrifuged in the Pegasus ultracentrifuge at 40K, at 3° C, for 90 minutes. After centrifuging, the supernatant from each tube was decanted carefully and measured spectrophotometrically for traces of protein. The concentration of the pelleted gel was measured or calculated (see section 2.3), and the tube cut to a spoon shape (Fig 2.1).

2.2.6 Gels of actin were also used at the lower concentration range of 25-65mg/ml. To prepare these, approximately 2-3ml of gel was placed in a small shallow container adapted from photographic film containers or from small jar-lids. The gel was then subjected to one of two alternative procedures. Some gels were sonicated, using a Rapidpol 400 ultrasonics unit and probe at the lowest setting for thirty seconds, then left to polymerize for a minimum period of twenty minutes before measuring. The remaining gels were stirred rigorously by hand and syringed or pipetted to ensure thorough mixing, and measured immediately.

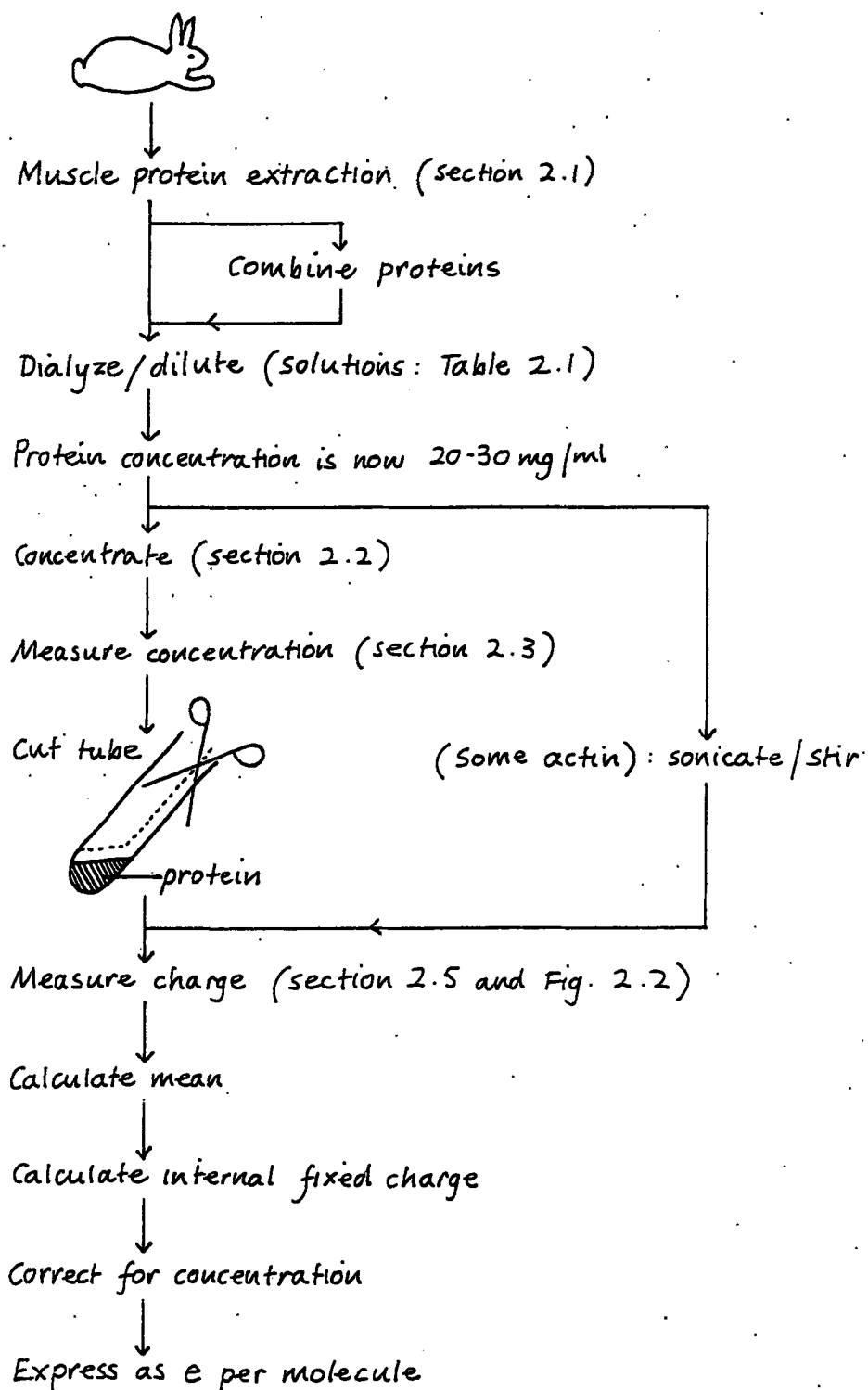


Figure 2.1 : Methods flowchart

## **Section 2.3 : Concentration Measurement of the Ultracentrifuged Gel**

2.3.1 Methods of concentration measurement which involve dissolving a known quantity of the substance to be measured in some other substance proved unsatisfactory because of the very small size of the sample. Two alternative methods were therefore used.

2.3.2 The first method, calculating concentration from precise and accurate weight measurements, had the advantage that no part of the pelleted gel was removed or disturbed. Before filling and centrifuging, the empty, labelled tubes and labelled caps were weighed. The concentration of protein in the solution to be centrifuged was pre-determined. After filling the tubes with this solution, and capping, each tube assembly was weighed again in order to calculate the total net volume of solution in the tube, and hence the total quantity of protein. After the tubes had been centrifuged and the supernatant decanted, each tube, with the pellet adhering to it, was weighed, and the net weight of the pellet was calculated. (A typical pellet weight was 0.05mg). After taking account of any protein found in the supernatant\*, the concentration of the pellet was calculated from the pellet weight and the total quantity of protein in the tube.

Results obtained in this way corresponded to within an error of not more than 5% with those obtained by the second method of concentration determination.

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\* In plain myosin and actin gels generally none was detectable, but in some protein combinations a proportion of the secondary protein was expected to remain unbound.

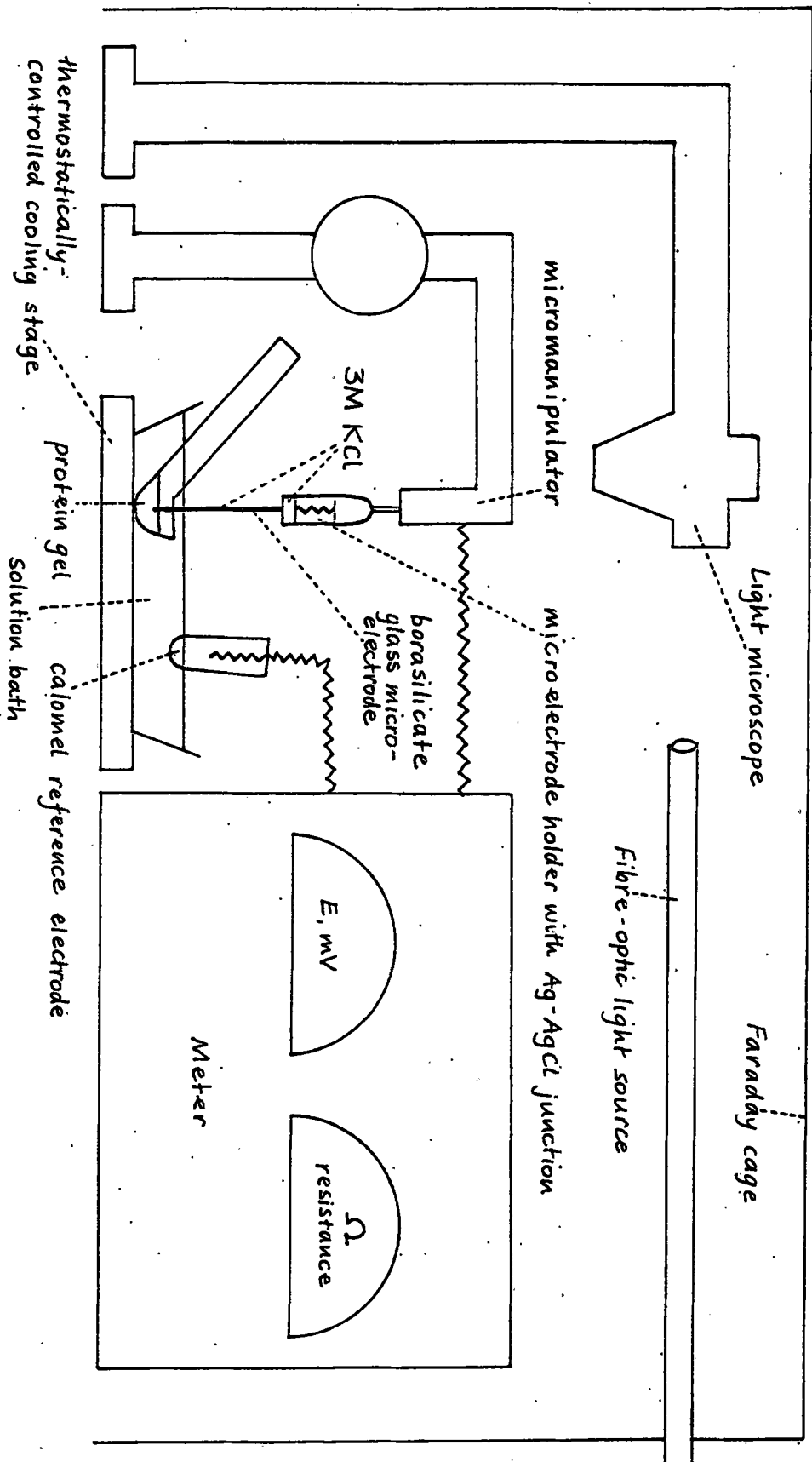
2.3.3 This second method was direct spectrophotometric measurement, using a 0.1mM sandwich cuvette. This method involved removing a tiny piece of the gel and flattening it between the two halves of the cuvette. It was then measured in the standard way, at A280 and A320, and the concentration calculated using an extinction co-efficient of 0.52 for myosin and 1.15 for actin (procedure standard in Dr C Moos' laboratory).

## **Section 2.4 : Microelectrodes**

2.4.1 The apparatus for measuring the gel potential is shown in Figure 2.2. During charge measurement the gel was immersed, as shown, in a solution of identical composition to that in which the protein was ultracentrifuged.

2.4.2 Microelectrodes were made using the standard microelectrode tubing and vertical puller (see Appendix to Section 2.4, below). For convenience, a batch of 20-30 microelectrodes was pulled and then stored, under glass to prevent accretion of dust, until needed. They were filled with 3M KCl; this was done immediately before use, rather than in advance, since it was found that the practice of batch filling and storage in KCl tended to result in crystallisation at the tips and on the exposed surface of the tubing. The microelectrode was mounted in a holder with a silver-silver chloride junction (see Appendix), also filled with 3M KCl.

2.4.3 Initial experiments took place using microelectrodes with tip diameters of about 0.5-1.0  $\mu\text{m}$  - the standard sharp microelectrode. In the gels, however, it was found that these clogged very easily, and frequently refused to give any reading at all. As a result, broken microelectrodes were tried (see Section 5.1). This followed experience of their use at Open University Summer Schools, where they were used



**FIGURE 2.2 :** Apparatus for measuring charge in protein gel



to check the reference potential of the circuit. These proved to give satisfactory and consistent readings.

2.4.4 To make them, normal sharp microelectrodes had a small section (usually less than 1mm) broken off the tip, using forceps. The tip sizes thus produced ranged from 20-100 $\mu$ m (exterior diameter); readings were consistent throughout this range. Under the light microscope the broken tips were observed to be typically jagged rather than straight.

To confirm that the consistency of readings was independent of microelectrode tip size, one experiment was conducted in which four microelectrodes were washed, dried, and their tip diameters recorded using a light microscope, following their insertion in the gel. Results are shown in Table 2.2.

#### APPENDIX to Section 2.4

Microelectrode tubing:	Clark Electromedical Instruments - borosilicate glass capillary tubing GC150F with internal fibre for back filling: length 65mm (before pulling), external diameter 1.5mm
Vertical Microelectrode puller:	Scientific & Research Instruments
Microelectrode holder:	Transidyne Corp - silver-silver chloride junction, model EH-1S
Reference electrode:	Corning Scientific Instruments
Micromanipulator:	Prior
MV and resistance meter:	Adrian Tuddenham, Meat Research Institute, Bristol, after Naylor 1978

**Faraday cages:**

**Open University, Oxford Research Unit, and Dr**

**Carl Moos, SUNY Stony Brook**

**Light microscope:**

**Zeiss**

Microelectrode external diameter, $\mu\text{m}$	61	68	47	42
Successive readings, mV	2.1; 2.8; 2.1; 2.2; 2.6; 2.5	2.6; 2.8	2.8; 2.6	2.5; 2.2
Mean	$n = 6$ $\bar{x} = 2.4$ $s = 0.3$	$n = 2$ $\bar{x} = 2.7$	$n = 2$ $\bar{x} = 2.7$	$n = 2$ $\bar{x} = 2.4$
Overall mean : $n = 12$ $\bar{x} = 2.4$ $s = 0.3$				

**TABLE 2.2 :** Diameters of four microelectrodes used for successive readings in myosin gel, 0.75A solution, pH 7.

### **Section 2.5 : Measuring the gel potential**

The cut tube containing the protein gel (Figs 2.1 and 2.2) was immersed in a bath of solution, matching the composition and ionic strength of the solution used during the preparation of the gel. During some experiments this bath rested on a thermostatically-controlled cooling stage; it was found, however, that the absence of a cooling system and the consequent slight rise in temperature of the solution during measurement did not appear to affect the consistency of readings; in most cases the measurements were completed within not longer than ten minutes. During this period the temperature of the gel, in the absence of a cooling stage, would usually rise to room temperature.

A calomel reference electrode also rested in the solution bath; a micromanipulator, connected to the microelectrode with a silver-silver chloride junction holder, was used to insert the microelectrode in the gel. The micromanipulator and the reference electrode were connected to the meter (as in Fig 2.2), thus completing the circuit.

Using a binocular light microscope for monitoring, the microelectrode was inserted about 25 times, into different parts of the gel, and readings of potential difference ( $E$ , mV) taken from the dial display on the meter. Where the resistance was observed to rise more than about  $2M\Omega$  the reading was discarded and the microelectrode replaced (see also Chapter 5, section 5.1). The readings were averaged and the standard deviation obtained. From the mean of the readings the internal fixed charge was calculated (see section 2.6) using the Nernst equation. External free ion concentrations used in this equation were calculated using a

computer program based on the Perrin equation (Perrin & Sayce, 1967), and using association constants given in Sillen & Martell, 1964 and 1971.

## **Section 2.6 : Calculation of internal fixed charge**

2.6.1 Most proteins carry a net negative charge at the normal intracellular pH, about pH 7, and it is this negative charge on the protein gel matrix which gives rise to the observed potential difference (E) between the gel and its bathing solution. The internal concentration of diffusable ions can be calculated from the Nernst equation:

$$E = \frac{RT}{ZF} \log_{10} \frac{[C]_o}{[C]_i} \text{ mV}$$

where R = gas constant

T = absolute temperature

Z = charge on ion

F = Faraday constant

[C]<sub>o</sub> = external ion concentration

[C]<sub>i</sub> = internal ion concentration

On the assumption that all ions are distributed independently of each other, this gives:

$$E = 58 \log_{10} \frac{[C]_o}{[C]_i} \text{ mV}$$

and

$$[C]_i = [C]_o \text{ antilog } \frac{E}{58}$$

The fixed charge on the gel can thus be calculated as the sum of the charges on the internal ions, derived from the concentrations.

2.6.2 An example is given in Table 2.3, where the free external charge has been calculated for the constituent ions of a particular solution, using the Perrin computer program, and the external fixed charge derived. In the example in Table 2.3,  $E=3.64$ . As in all results, this is the mean of at least fifteen readings (in this case 25), obtained by microelectrode insertion into different parts of a gel (in this case in the standard 0.5A solution).

The constants, derived from the Nernst equation, are used as follows:

$$[Pr]_{\text{charge on monovalent positive ion}} = [C]_o \times \text{antilog } \frac{E}{58}$$

$$[Pr]_{\text{charge on divalent positive ion}} = [C]_o \times \text{antilog } \frac{E}{29}$$

$$[Pr]_{\text{charge on monovalent negative ion}} = [C]_o \div \text{antilog } \frac{E}{58}$$

$$[Pr]_{\text{charge on divalent negative ion}} = [C]_o \div \text{antilog } \frac{E}{29}$$

Microelectrode readings in 0.5A actin gel, 130 mg/ml

3.9	3.4	3.3	4.7	3.3	3.5	3.5	3.3	3.6
3.4	4.4	4.4	3.2	3.3	3.5	3.5	4.0	5.8
3.9	3.2	3.3	3.3	3.8	3.9	3.6		
<hr/>								
n = 25	$\bar{x}$ = 3.64 mV	$s = 0.16$						

Table 2.3a

$$R_{\text{monovalent}} = \text{antilog} \frac{3.64}{58} = 1.1555$$

$$R_{\text{divalent}} = \text{antilog} \frac{3.64}{29} = 1.3351$$

Table 2.3b

Ion	[C] <sub>o</sub>	[C] <sub>i</sub>	[Pr]
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K <sup>+</sup>	65.36	75.52	75.52
Cl <sup>-</sup>	55.00		
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	3.87		
KHPO <sub>4</sub> <sup>-</sup>	0.74		
total monobasic negative	59.61	51.59	51.59
Mg <sup>++</sup>	1.10	1.47	2.94
HRO <sub>4</sub> <sup>==</sup>	0.74	0.55	1.11

$$\text{net negative charge @ 130 mg/ml} = 25.76e$$

$$\text{@ 100 mg/ml} = 19.82e$$

$$\text{actin e/monomer (from Table 2.4)} = 8.3 \pm 0.4$$

Table 2.3c

Table 2.3 : stages in the calculation of charge per monomer : a worked example

Protein	Conversion constant
Myosin	4.78
Actin	0.42
Tropomyosin-troponin	1.45
Myosin rod	2.2
Myosin S-1	1.3
C-protein	1.4

Table 2.4: Conversion constants for muscle proteins,  
based on molecular weights.



2.6.3 The net fixed charge is more usefully expressed as e per molecule. To convert to this, it is convenient to employ two stages. In the first, the measured gel concentration is used to calculate and express the fixed charge which would be found in the protein at 100mg/ml ( $\text{Pr}^-/\text{mM}$ ). This figure can then be converted by multiplying by a constant for each protein (Table 2.4). The constant is the molecular weight of each protein (sections 1.6 - 1.9 above)  $\times 10^{-5}$ . The final figure in the example in Table 2.3 is calculated in this way (see Chapter 1, sections 1.6-1.9).

## **Section 2.7 : Errors**

2.7.1 Where sufficient experiments relating to a given condition were carried out, the results include the standard error on the mean (sem). Where more than three but fewer than ten experiments took place the standard deviation (6, or sd) is shown, but no standard error. Where there are fewer than three experiments an estimated error (ee) is shown. The estimated error is derived by comparison with the standard deviation found under similar conditions; a figure of at least 2 x the comparable standard deviation is given.

2.7.2 Sources of intrinsic error are likely to be instrumental limitations, human and personal error, and variations in biological material. Instrumental limitations include the possibility of errors in measuring very small quantities of materials on a chemical balance or in a glass syringe, as well as possible systematic errors introduced into the calibration of the meter or of other pieces of electronic equipment. These may contribute to an error of up to 1% each. Human and personal error includes parallax error and similar small misjudgements in the composition of solutions, an over- or under-estimate of readings, and possible errors of judgement in discarding or retaining apparently anomalous readings. These may

contribute to an error of up to 5%. Small variations in biological material are an inevitable concomitant of all biological systems. The error contributed is perhaps 5%. The total error, therefore, is likely to be in the order of 15%.

## CHAPTER 3

### RESULTS OF CHARGE MEASUREMENTS

#### Section 3.0 : Introduction

3.0.1 The charge on all the proteins examined was measured in the A series of solutions (Table 2.1), to establish the extent of their ionic strength-dependence. A larger number of measurements was made in solution A, as this also served as a control for other experimental conditions.

3.0.2 In addition to the A series, a number of experiments was performed in which the chemical composition of the solution was altered, in order to observe the effect of the addition of particular substances (eg ATP) or the presence or absence of particular ions. In all cases alterations were made both to the composition of the gel and to the bathing solution in which it was measured; except in one experiment (with phosphatase, Section 3.1.9), gels were not re-equilibrated in new, different solutions. The nearest analogous A solutions were used as controls.

3.0.3 The charge on myosin was measured in four solutions from the experimental A series (3.1.3). Myosin rod was also measured in this series (3.1.5). C-protein was combined with myosin and with myosin rod and was measured in A solution, to observe the effect on the myosin charge. The effects of the presence of ATP, ADP, DAPP (3.1.6 - 3.1.8) and PPi (3.1.11) were tested, as were the effects of the omission of chloride and magnesium. Myosin was also phosphorylated and measured with and without phosphatase (3.1.10).

3.0.4 Ultracentrifuged F-actin\* was measured in four A solutions (3.2.1) and in a solution containing PPI (3.1.10). It was also combined with the S-1 portion of myosin (3.2.5) and with tropomyosin-troponin (3.2.1). In the latter case the effects of the presence of calcium and of EGTA were also tested (3.2.4). Low-concentration F-actin gels were also measured under stirred, rested and sonicated conditions.

3.0.5 Actomyosin was measured in the A series (3.3.1) and in the presence of PPI (3.3.2).

### **Section 3.1 : Myosin**

3.1.1 Results are shown both as a charge concentration (Pr-/mM) at 100mg/ml\*\* and as electrons per molecule (e/mol)\*\*\*. Error figures are shown as standard error (se), standard deviation (sd) or estimated error (ee); see Section 2.7.1.

3.1.2 Table 3.1 shows both Pr-/mM and e/mol for both actin and myosin, in the standard experimental solutions. While myosin has the higher charge per molecule, actin, being a smaller molecule than myosin, has a higher charge per unit volume.

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\* Unless otherwise indicated, the term actin refers throughout to F-actin.

\*\* This is the calculated concentration of charge (normally negative), in mM, which would be found in a protein concentration of 100mg/ml. See section 2.6.

\*\*\* Where more than one protein is present in a gel, it is not always possible to derive a charge per molecule for each of them, separately. See Section 5.2.

3.1.3 In the main A series, the effect of ionic strength (I) on the (negative) charge per myosin molecule is shown in Table 3.2, and Fig 3.2. It will be seen that the charge is relatively constant at ionic strengths 69 - 140mM but that it falls away sharply at  $I = 29\text{mM}$ .

3.1.4 An analysis of the concentration range of the A pellets showed that there was little correlation between concentration and measured charge (Fig 3.1). This contrasts with the actin results, which demonstrate a significant correlation (Fig 3.3). This is discussed in Section 3.2 below.

3.1.5 The effect of ionic strength on the charge per molecule of the rod section of the myosin molecule, separated by standard digestion methods (Section 2.1.6) is shown in Table 3.3 and Fig 3.2. In these experiments the charge on myosin rod appears to be closely related to ionic strength. The rod charge appears to be a high proportion of the whole myosin charge at higher ionic strengths; it seems, however, to fall to only 31% of the whole in 0.2A. This is a puzzling result when taken in conjunction with the results for the S-1 fragment, the other part of the total myosin molecule (see section 3.2.9): this appears also to decrease in charge as ionic strength is lowered and, indeed, gives a small positive charge in 0.2A. In other words, the charge found on the whole (intact) myosin molecule is not entirely consistent with the sum of the parts found in these experiments (see Section 5.7.1).

Solution	Myosin Pr <sup>-</sup> /mM @100 mg/ml	Myosin e/mol	Actin Pr <sup>-</sup> /mM @100 mg/ml	Actin e/mol
A	14.6	70 ± 3 (se)	21.4	9 ± 1 (se)
0.75 A	14.6	70 ± 13 (sd)	19.0	8 ± 1 (se)
0.5 A	14.8	71 ± 26 (ee)	19.0	8 ± 1 (se)
0.2 A	10.0	48 ± 13 (sd)	14.3	6 ± 3 (sd)

Table 3.1 : Comparative results for actin and myosin, shown as Pr<sup>-</sup>/mM at 100 mg/ml and as e/molecule.

Throughout the Tables, error figures are shown as one of three types: standard error (se); standard deviation (sd); and estimated error (ee). See Section 2.7.1.

Solution	I, mM	e/mol	ee	sd	se	concentration range, mg/ml	n
A	140	70	-	12	3	138-274	14
0.75 A	105	70	-	13	-	84-187	4
0.5 A	69	71	26	-	-	85-208	3
0.2 A	29	48	-	13	-	100-217	5

Table 3.2 : Myosin in A solutions

I = ionic strength.

n = number of experiments

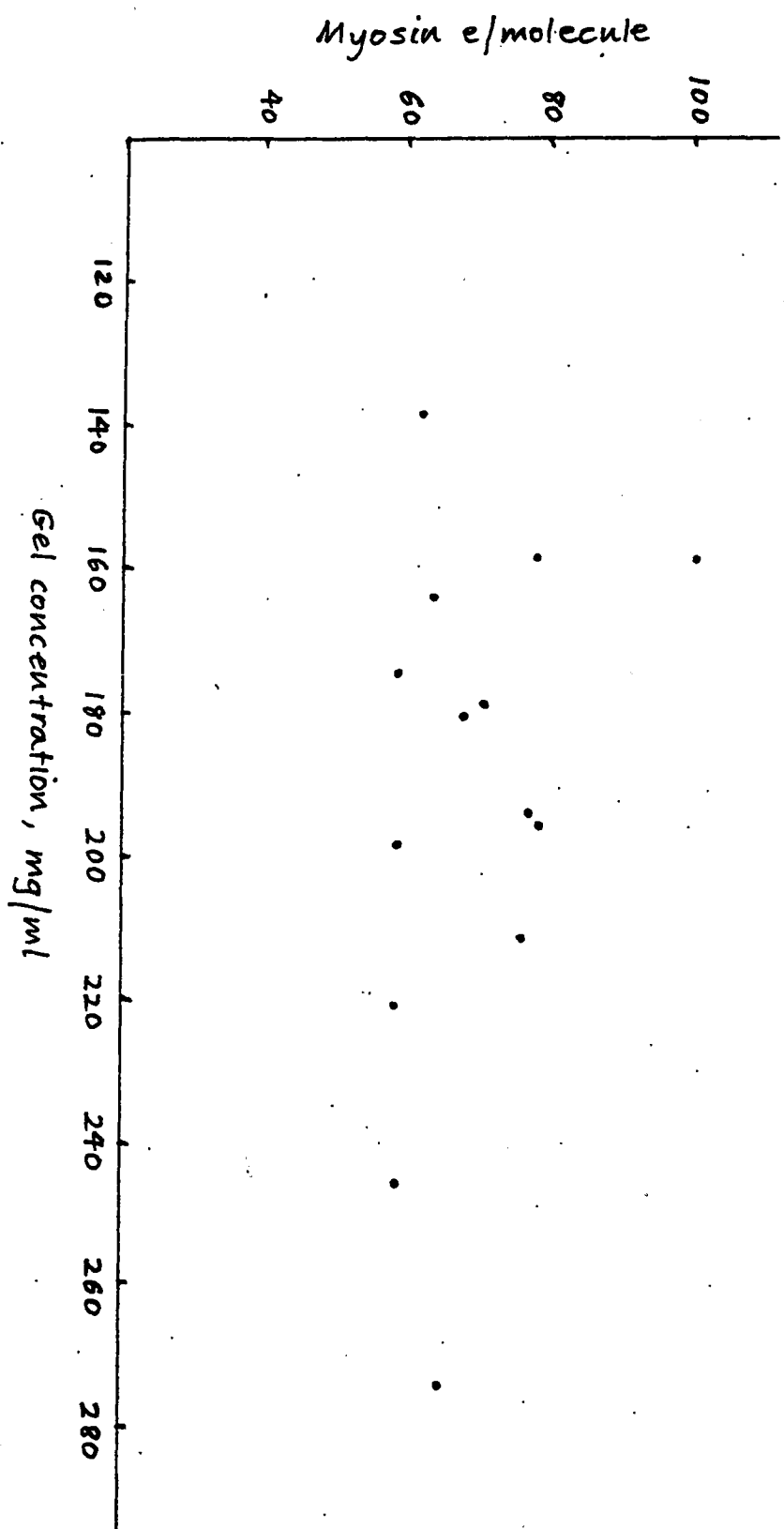


Fig. 3.1 : myosin in A solution : charge vs. concentration

Solution	I, mM	e/mol	ee	sd	% of myosin charge	n
A	140	53		10	75%	3
0.75 A	105	66	20	-	94%	1
0.5 A	69	51	20	-	72%	1
0.2 A	29	15	7	-	31%	2

Table 3.3: Myosin rod in A solutions

% C-protein in gel	Pr <sup>-</sup> /mM @ 100 mg/ml (total protein)	e/mol myosin	ee
27%	20	131	20
22%	23	141	21
19%	25	148	22
14%	26	145	22

mean = 140

Table 3.4: Myosin + C-protein in A solution, I = 140 mM.  
This table shows results of four experiments under identical conditions.



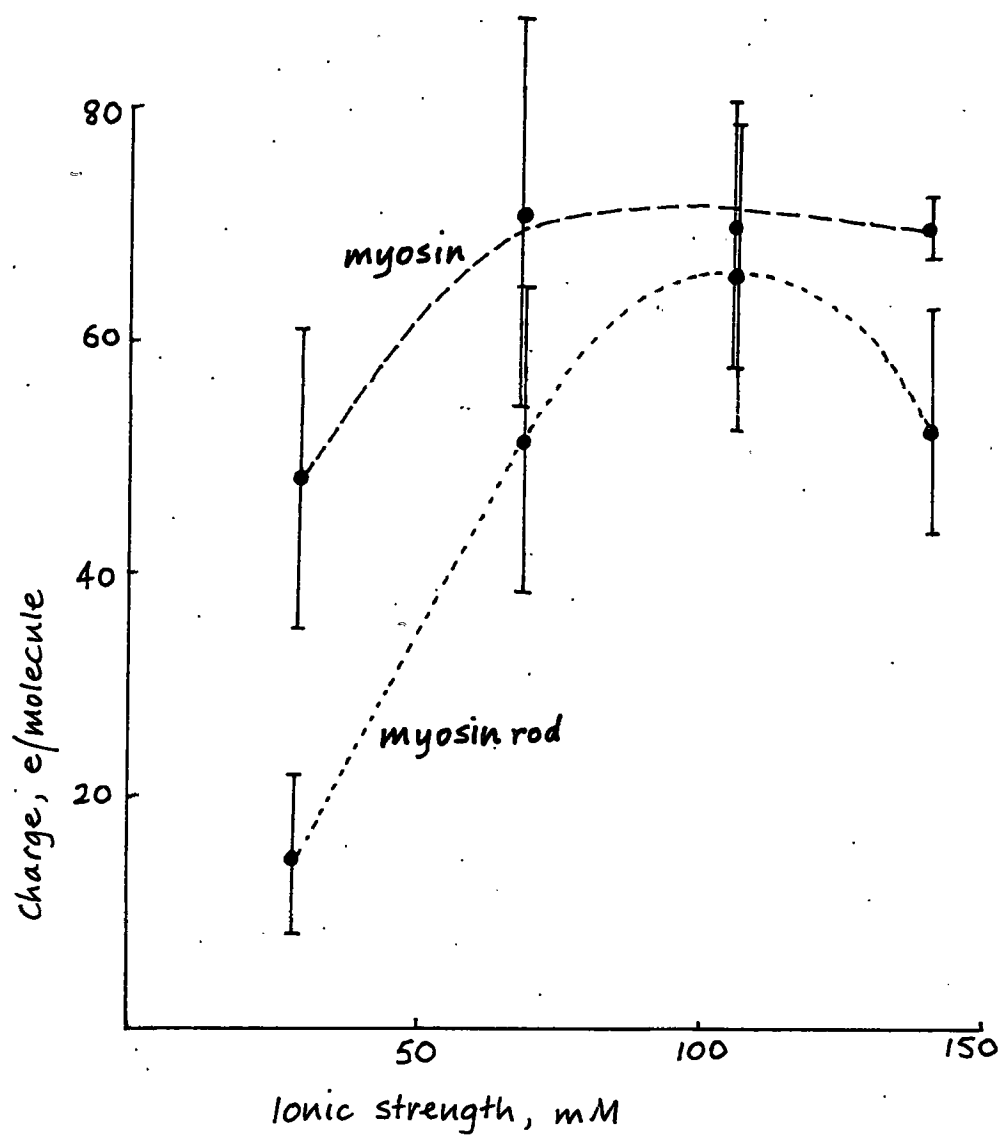


Figure 3.2 : Myosin and myosin rod in A solutions.  
(From Tables 3.2 and 3.3.)

In addition to these simple ionic strength experiments, the effect on the myosin charge of the addition of various substances was tested in A solution.

3.1.6 The addition of C-protein to myosin (Table 3.4) appears to double the charge to 140e (mean of four experiments); this calculation is made on the assumption that the C-protein itself contributes zero net charge, but alters the charge of the myosin molecule (but see discussion in section 5.2). The addition of ATP to myosin + C-protein (Table 3.5) produced no change (one experiment). Myosin rod was measured with C-protein in A solution (Table 3.6), the charge on the rod being increased to 166e per molecule, again approximately double the charge found in the absence of C-protein.

As excess C-protein was used in the protein solution before ultracentrifuging, the amount of C-protein contributing to the final gel was calculated by measuring the amount left in the supernatant. The percentage of C-protein in the gel was found to vary considerably, and is therefore shown in Tables 3.4 - 3.6. In Table 3.5 a single gel of myosin + C-protein is shown, that containing 27% C-protein, since it is the gel most comparable with the myosin-C-protein-ATP gel, which contains 29% C-protein.

For alternative interpretations of the C-protein results, see Chapter 5, section 2.

3.1.7 In myosin alone, the presence of ATP (5mm MgATP) appears to increase the charge: a 51% increase was measured, in five experiments, with a mean of 106e. ADP (5mm) increases it still further, to 135e (Table 3.7), an increase of 93%. In the presence of 10  $\mu$ m myokinase inhibitor diadenosine pentaphosphate (DAPP), in addition to ADP, the charge was 115e (two experiments). DAPP would be expected

	$\text{Pr}^-/\text{mM}$	$\text{e/mol}$ myosin	% C-protein	n
Myosin	15	$70 \pm 3$ (se)	-	14
Myosin + C-protein	20	$131 \pm 20$ (ee)	27	1
Myosin + C- protein + ATP	20	$135 \pm 21$ (ee)	29	1
Myosin + ATP	22	$106 \pm 10$ (sd)	-	5

Table 3.5 : Effect on myosin charge of the presence of ATP and of C-protein, in A solution,  $I = 140$

$I, \text{mM}$	% C-protein	% myosin rod	$\text{Pr}^-/\text{mM}$ @ 100 mg/ml	myosin rod $\text{e/mol}$
140	43	57	30	$116 \pm 17$ (ee)

Table 3.6 : Myosin rod + C-protein in A solution, one experiment

	e/mol	n
Myosin + ATP	$106 \pm 10$ (sd)	5
Myosin + ADP	$135 \pm 13$ (sd)	3
Myosin + ADP + DAPP	$115 \pm 25$ (ee)	2
Myosin + DAPP	$76 \pm 14$ (ee)	1
Myosin (control)	$70 \pm 3$ (se)	14

Table 3.7: Myosin charge in the presence of ATP; ADP; and DAPP, in A solution,  $I = 140$  mM.

	I, mM	e/mol	ee	gel mg/ml	control e/mol	% increase
No chloride: K <sup>Ⓟ</sup> , K = 120 mM	144	$118 \pm 24$		124	70	69
Low chloride: Above + 5 mM MgCl <sub>2</sub>	144	$85 \pm 18$		154	70	21
No chloride: K <sup>Ⓟ</sup> , K = 24 mM	30	$104 \pm 21$		143	48	117
Low chloride: above + 5 mM MgCl <sub>2</sub>	31	$60 \pm 12$		167	48	25

Table 3.8: Myosin charge, with chloride reduced and with chloride absent. Control solutions were A ( $I = 140$  mM) and 0.2 A ( $I = 29$  mM)

to add 5-10e, contributed by the two pentaphosphates, on the assumption that it binds in stoichiometric ratio: this is irrespective of its effect, if any, upon the myosin charge. In all these experiments the substance in question (eg ATP) was present both in the composition of the gel and in the bathing solution.

3.1.8 Additions to, or other departures from, the chemical composition of the standard A solutions might be expected to affect the charge found on the molecule. In practice, however, it is rarely possible to standardise the chemical parameters, since altering one variable usually also alters either the ionic strength or the pH of the solution. Since changes to the ionic strength or the pH would on their own be expected to result in some change to the charge, it would not in most cases be possible to establish with confidence which variable was producing any observed effect. Re-adjusting the ionic strength or the pH would in turn introduce new changes, and thus possible charge effects, to the chemical composition of the solution.

3.1.9 However, these parameters were held relatively constant in a set of experiments in which chloride was absent from the solution, being replaced by additional phosphate in order to maintain a comparable ionic strength. The results (Table 3.8) show a marked increase in charge when the major co-ion is phosphate, in the absence of both chloride and magnesium, with a lesser, but still significant, increase in the presence of 5mM magnesium chloride. These solutions also included 20% additional potassium, but this is unlikely by itself to account for the increase in charge, since in one experiment in solution 1.5A, in which KCl was 150mM, the charge was only 80e.

3.1.10 Myosin was phosphorylated with myosin light chain kinase (MLCK), and was measured with and without C-protein; the phosphorylated gels were then re-equilibrated for 24 hours in phosphatase solution and measured again. In both cases the charge was reduced (Table 3.9). It is noticeable that while phosphorylated myosin has a significantly higher charge than normal myosin, phosphorylated myosin with C-protein has a lower charge than the very high level observed in normal myosin + C-protein. However, the percentage of C-protein which was found in the phosphorylated myosin gel was considerably lower than the percentage in the standard gels. The reason for this is not known, but could be investigated in further experiments.

3.1.11 The addition of PPi, Pyrophosphate (sodium pyrophosphate  $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ ), to the standard A and 0.2A solutions lowers the pH of the solution quite markedly, and raises the ionic strength (see Section 3.1.8). Limits are therefore imposed on the interpretation of the results, but it is interesting to note that under these conditions the charge on myosin (and on actomyosin: see section 3.3.2) appears to be increased in 0.2A but not in A (Table 3.10). It will be apparent that in 0.2A + PPi the myosin charge is raised to a level approximating that in A and 0.5A (Table 3.2). The ionic strength, 64mM, is approaching that of 0.5A solution, 69mM. The most probable explanation is that the charge increase is a simple ionic strength effect; this is also consistent with the absence of a charge increase in A + PPi, since beyond this level a higher ionic strength does not produce a markedly higher charge (one experiment with myosin in 1.5A,  $I=210\text{mM}$ ,  $e/\text{mol}=80$ ). However, this does not account for the absence of an effect on actin at low ionic strength.

3.1.11 Comparative myosin results are shown in Table 3.21.

	$\text{Pr}^-/\text{mM}$	$\text{e}/\text{mol}$	After phosphatase:		% C-protein
			$\text{Pr}^-/\text{mM}$	$\text{e}/\text{mol}$	
Phosphorylated myosin	24	$115 \pm 20(\text{ee})$	22	$105 \pm 18(\text{ee})$	-
Phosphorylated myosin + C-protein	23	$119 \pm 23(\text{ee})$	17	$89 \pm 18(\text{ee})$	7

Table 3.9 : Effect of phosphorylation on myosin charge in the presence and absence of C-protein. The myosin charge is calculated on the assumption that C-protein has a zero net charge, see Section 5.2.

$n = 1$  in each case.

	$\text{I}, \text{mM}$	$\text{pH}$	Myosin		Actin	$\text{e}/\text{mol}$	$\text{ee}$	Predicted actomyosin		Observed actomyosin	
			$\text{e}/\text{mol}$	$\text{ee}$				$\text{Pr}^-/\text{mM}$	$\text{ee}$	$\text{Pr}^-/\text{mM}$	$\text{ee}$
A	140	7.0	$75 \pm 18$		$8 \pm 3$			17		$17 \pm 6$	
A+10mM PPI	174	6.2	$66 \pm 17$		$8 \pm 4$			15		$17 \pm 6$	
0.2A	29	7.0	$43 \pm 11$		$8 \pm 4$			11		$10 \pm 5$	
0.2A+10mM PPI	64	5.6	$66 \pm 17$		$5 \pm 3$			14		$21 \pm 12$	

Table 3.10 : Effect on myosin charge of the presence of 10mM PPI, in A and 0.2A solutions.

$n = 2$  in each case.

## **Section 3.2 : Actin**

3.2.1 In the main A series, the effect of ionic strength on the charge per actin monomer is shown in Table 3.11. It is noticeable that though the actin charge falls somewhat with ionic strength, it does not reproduce the sharp fall seen in myosin at  $I = 29\text{mM}$ .

This table shows the combined results from measurements both of ultracentrifuged gels (concentration range 74-265 mg/ml) and of gels which were measured after the A solution dialysis stage (concentration range 23-75 mg/ml) (see Section 3.2.2).

Ultracentrifuged gels alone are shown in Table 3.12.

3.2.2 Measurements, principally in the two solutions 0.75A and 0.5A, were made in gels which had not been ultracentrifuged: these results are shown in Table 3.13.

The measurements of electrical potential in these latter gels were exceedingly consistent and reproducible; for example, 29 readings in one 0.5A gel were within the range 1.1-1.4mV, giving a standard deviation of 0.07.

3.2.3 The variations between the means of different experiments in this series were found to relate to the final stage of preparation of the gel. Gels which were sonicated or were left undisturbed for a period (4-24 hours) were found to have a significantly lower charge than gels which were hand-stirred or syringed immediately before measuring, as shown in Table 3.14.



Solution	I, mM	e/mol	sd	se	gel mg/ml	n
A	140	9	4	1	65-228	15
0.75A	105	8	4	1	24-265	11
0.5A	69	8	4	1	23-186	15
0.2A	29	6	3	-	26-151	9

Table 3.11 : Actin in A solutions

Solution	I, mM	e/mol	sd	se	gel mg/ml	n
A	140	9	3	1	74-228	13
0.75A	105	9	5	-	122-265	4
0.5A	69	8	5	-	108-186	5
0.2A	29	6	4	-	80-151	8

Table 3.12 : Ultracentrifuged actin in A solutions

Solution	I, mM	e/mol	sd	se	gel mg/ml	n
0.75 A	105	7	1	-	24-56	7
0.5 A	69	9	3	1	23-75	10

Table 3.13 : Actin after dialysis in A solutions; not ultracentrifuged

Solution	I, mM	sonicated:			stirred:			difference:	
		e/mol	sd	n	e/mol	sd	n	e/mol	+%
0.75 A	105	6 ± 1	3		8 ± 1	4		2	38
0.5 A	69	8 ± 3	7		11 ± 2	3		3	38

Table 3.14 : Comparison between sonicated and stirred actin gels in 0.75A and 0.5A

Solution	I, mM	undisturbed:		stirred:		+%
		e/mol	mg/ml	e/mol	mg/ml	
0.75A	105	6	258	8	265	33

Table 3.15 : Actin gels rested or stirred before ultracentrifuging, one experiment

This shows a remarkable 38% increase in charge after stirring, in a very consistent set of results. There was some variation between the charge found in different batches of actin or sets of experiments, but in each case the charge after stirring or syringing was raised by between 30% and 50%. The two effects could be produced repeatedly in succession in the same gel. A t-test run on the measurements of potential for the rested and the stirred states of one actin gel in 0.75 A was significant at the 0.01 level : that is, there is less than a 1% probability that this result could have occurred by chance.

3.2.4 This phenomenon was also demonstrated in one experiment with ultracentrifuged actin, in which two tubes of actin were used. One tube was filled, sealed and left overnight without being disturbed; the following day the second tube was filled with vigorously-stirred and syringed actin from the same batch, and both tubes were then immediately ultracentrifuged in the standard way. The result is shown in Table 3.15.

It is likely that this effect accounts to a significant extent for the relatively high standard deviation between results in all other ultracentrifuged actin gels: these were made and measured before this effect was discovered, and were therefore not monitored for it.

3.2.5 A hypothesis which was considered at an early stage in the ultracentrifuged experiments, but rejected, was that the charge on actin declined as the preparation aged, over a period of about a week after extraction from actin powder (this period is the limit of the life of the preparation). It may be that the apparent correlations which tended initially to support this hypothesis were in fact an unrecognised effect of allowing the gel to rest. These findings all point to the likelihood that, in actin,

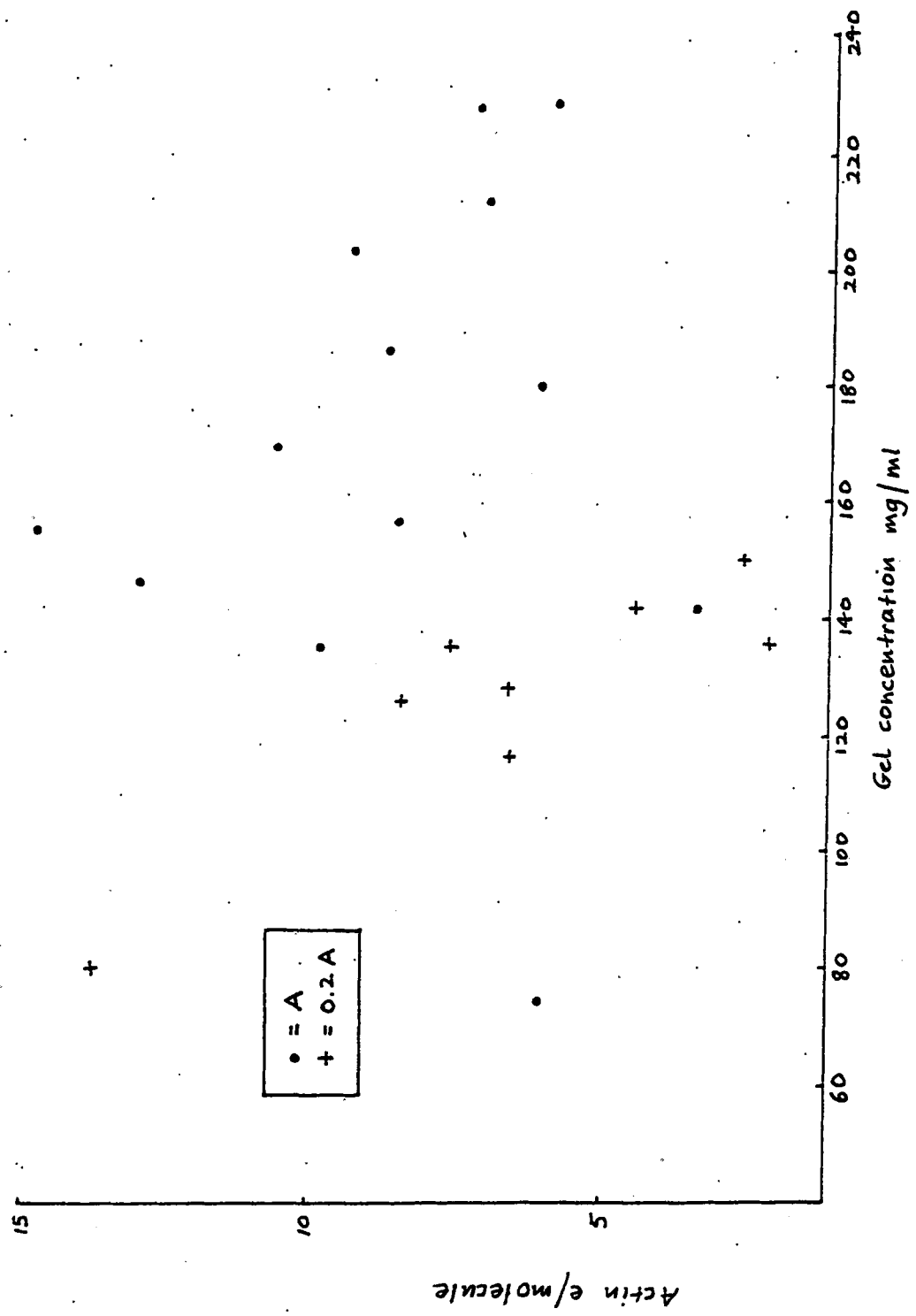


Fig. 3.3: ultracentrifuged actin in A and 0.2A solutions: charge vs. concentration

level of charge is affected by the state of aggregation of the gel. It is possible that there may also be some correlation between the concentration of the ultracentrifuged gels and their charge, with gels of higher concentrations tending to show a lower charge within a range of gels of identical ionic strength. These are shown in Fig 3.3. However, the points are relatively scattered and the number of samples too small to draw any positive conclusion; further work with a larger number of experiments, and statistical analysis, would show whether this was a real effect or simply a random impression. If the effect were found to be demonstrated, it would contrast with the pattern of myosin gels (Fig 3.1) which do not give any indication of any correlation of this kind.

3.2.6 Fig 3.3, in which the concentration of ultracentrifuged actin gels is plotted against their charge, shows that in these gels lower ionic strength is accompanied by lower (average) concentration, despite all gels being subjected to the same centrifugation conditions. This appears to be a demonstration of the phenomenon called the screening effect (see Appendix), described by Verwey & Overbeek (1948) and shown in the work of Rome and others (e.g. Rome 1967): as the concentration of an ionic solution is decreased, the electrostatic repulsion between suspended charged particles increases. This means that the particles move further apart from each other, thus giving a lower concentration.

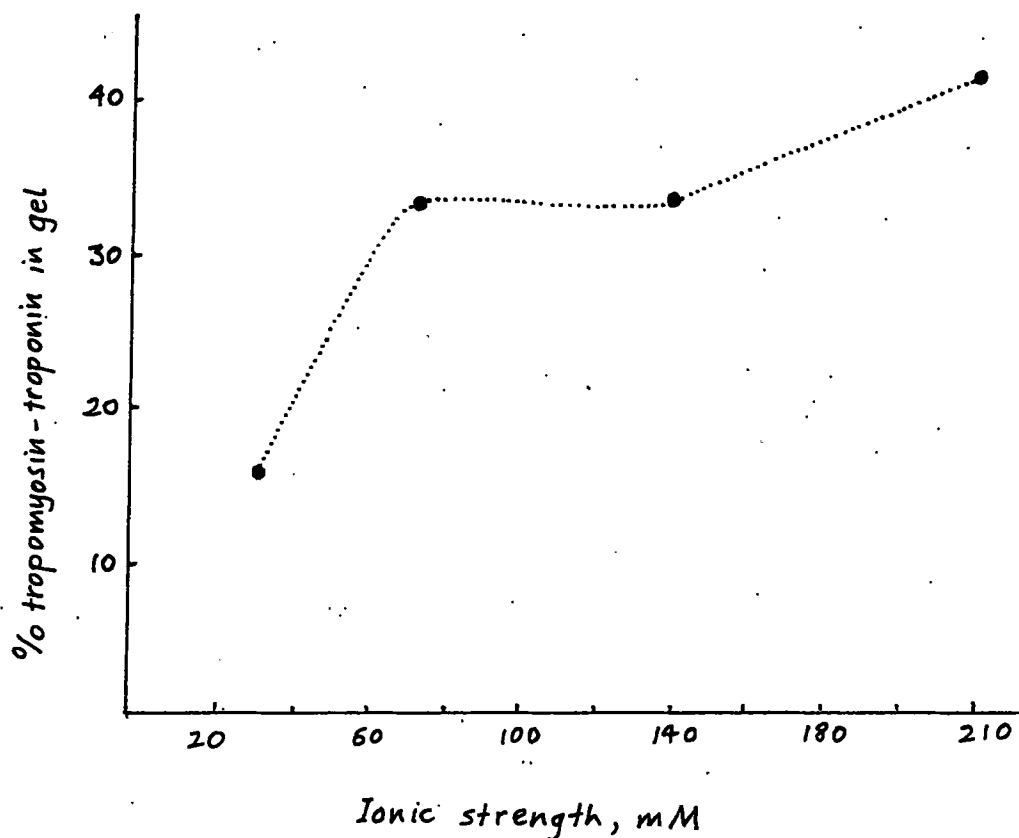


Fig. 3.4: Effect of ionic strength on the percentage of tropomyosin-troponin in the actin-t-t gel. The stoichiometric percentage is about 33%, or actin: tropomyosin-troponin :: 2:1.

Solution	Pr <sup>-</sup> /mM	e/monomer (see text)
1.5A	23	15
A	47	31
	23	15
} mean = 23 ± 12 (ee)		
0.5A	45	25
0.2A	25	19
	26	21
	32	20
} mean = 20 ± 6 (ee)		

Table 3.16: Actin-tropomyosin-troponin charge, all experiments

3.2.7 Actin was combined with tropomyosin-troponin 1:1 by weight in the expectation that the proteins would bind in the ratio 2:1\*, with excess tropomyosin-troponin remaining in the supernatant after ultracentrifuging. This ratio was indeed found in A and 0.5A solutions. In 1.5A more tropomyosin-troponin was retained in the gel, at a ratio of about 3:2, perhaps as a result of the strong self-associating propensity of tropomyosin. In 0.2A much less was retained, leaving a ratio of between 4:1 and 5:1: this was confirmed in three separate experiments. (The binding curve is shown in Fig 3.4). In the 0.2A result, therefore (Table 3.16), the charge attributable to free actin (that is, actin which is not complexed with tropomyosin-troponin) was calculated and subtracted from the total protein charge before the charge for the actin-tropomyosin-troponin complex was calculated. In the case of 1.5A it was not possible to make an equivalent adjustment, in this case subtracting a free tropomyosin-troponin charge, since there was no independent experimental measure of such a charge; however, if the charge is calculated from the amino-acid sequence of tropomyosin-troponin, the error on the uncorrected figure is likely to be only about  $2e/mol$ .

The calculation of the  $e/monomer$  charge for the actin-tropomyosin-troponin complex is based on actin + one-seventh\*\* of the tropomyosin-troponin complex.

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\* This is the weight ratio of actin:tropomyosin-troponin.

\*\* While the actin-tropomyosin-troponin weight ratio is 2:1, there are seven actin molecules for each molecule of tropomyosin-troponin, and it is this latter ratio which is applicable to the charge per monomer.

Solution	Actin e/mol	Actin+ EGTA e/mol	Actin- t-t Pr <sup>-</sup> /mM	Actin- t-t e/mon	Actin-t-t +EGTA Pr <sup>-</sup> /mM	Actin-t-t +EGTA e/mon
A	10±3	13±3	23±5	15±3	35±7	15±4
0.2A	6±2	10±3	26±6	21±5	35±7	26±6

Table 3.17 : Charge on actin molecule and on actin-tropomyosin-troponin monomer, in the presence/absence of EGTA.

All errors shown are ee. In each case  $n = 1$ .

Solution	Actin Pr <sup>-</sup> /mM	Actin-S-1 Pr <sup>-</sup> /mM	Derived S-1 Pr <sup>-</sup> /mM	Derived S-1 e/mol
A	36±8	19±5	9	10
0.75A	42±9	17±5	5	6
0.5A	38±8	11±3	~0	~0
0.2A	33±7	6±2	+3	+3

Table 3.18 : Actin + S-1, showing the derived net charge on S-1 (see Section 3.2.5).

All errors shown are ee. In each case  $n = 1$ .



3.2.8 The effect of calcium on actin-tropomyosin-troponin was tested by including 0.1mM CaCl in gels in A and 0.2A solutions: no effect was observed, the readings in each case being virtually identical with those in the control gels.

To test whether, on the other hand, chelating any already-bound calcium would produce a change in charge, 1mM EGTA was included in gels in A and 0.2A (Table 3.17). This shows a significant increase in charge with the addition of EGTA, with a particularly large effect in the lower ionic strength solution.

3.2.9 The S-1 subfragment of myosin was combined with actin in order to measure the charge characteristics of S-1 (on its own, S-1 will not cross-link to form a gel). The charge found on the control actin gel has been subtracted from the total charge measured in the actin-S-1 gel in order to derive a net charge for S-1 alone. Results are shown in Table 3.18, and support the supposition that the majority of the myosin charge derives from the rod fragment (but see also section 3.1.5).

It will be seen that this experiment showed that at low ionic strength S-1 carries a small positive charge as predicted from the amino acid sequence data by Naylor et al, 1985. In Table 3.19 the charges found on myosin rod (section 3.1.5) and on S-1 have been added together and compared with the charge found on intact myosin. In A solution the comparison is good, but at low ionic strength there is a considerable discrepancy. This is discussed in Section 5.7.1.

3.2.10 The effect of the addition of 10mM pyrophosphate, with a discussion of the limitations of interpretation of the results, is shown in section 3.1.10 and Table 3.10.

3.2.11 Comparative actin results are shown in Table 3.22.

Solution	i myosin rod e/mol (Table 3.3)	ii S-1 e/mol x 2 (Table 3.18)	i + ii Total of rod + S-1 charge	Intact myosin (Table 3.1)
A	53	20	73	70
0.75A	66	12	78	70
0.5A	51	~0	51	71
0.2A	15	+6	9	48

Table 3.19: Comparison of the charge on myosin rod, myosin S-1, and intact myosin.

Errors not shown.

### **Section 3.3 : Actomyosin**

3.3.1 Gels of actomyosin proved very difficult to measure: the gel itself appeared sticky in consistency and was therefore liable to clog the microelectrodes; and there were other curious features not found in gels of myosin or actin, see section 3.3.3 below.

Where it was possible to be confident of the reliability of measurements, the results varied. The preliminary result suggested an increase of 100% over the charge predicted by the contribution of each of the two proteins separately. Later results, however, did not reproduce this, confirming, rather, that actomyosin charge is no greater than the sum of the myosin and actin charges (Table 3.20).

3.3.2 The effect of the addition of pyrophosphate to actomyosin is shown in Table 3.10 (above, in Myosin section). At low ionic strength the charge on actomyosin is considerably increased, in the same way as myosin but not actin. It is reasonable to suppose that the effect seen in actomyosin is in fact an effect being produced in the myosin part of the actomyosin complex.

3.3.3 There were two curious features of actomyosin measurements which were not found in myosin or actin. In some experiments with actomyosin, two distinct peaks of readings emerged. The readings from one such experiment are shown in the form of a histogram in Fig 3.5.

Secondly, on some occasions, if the microelectrode was allowed to remain in the gel for a few seconds after insertion, the initial reading would be followed by a perceptible, though gentle, rise or fall to a second reading, which then remained

steady. It seems likely that these two features are linked: where both occurred within the same experiment, the movement was from one of the two discrete ranges to the other. Possible interpretations are discussed in section 5.8.

Solution	Control myosin:		Control actin:		Predicted actomyosin	Observed actomyosin	n
	Pr <sup>-</sup> /mM	e/mol	Pr <sup>-</sup> /mM	e/mol	Pr <sup>-</sup> /mM	Pr <sup>-</sup> /mM	
A	15	72 ± 14	21	9 ± 2	17 ± 4	18 ± 5	3
0.2A	9	43 ± 10	17	7 ± 2	11 ± 3	10 ± 2	2

Table 3.20: Actomyosin in A and 0.2A solutions.

All errors shown are ee.

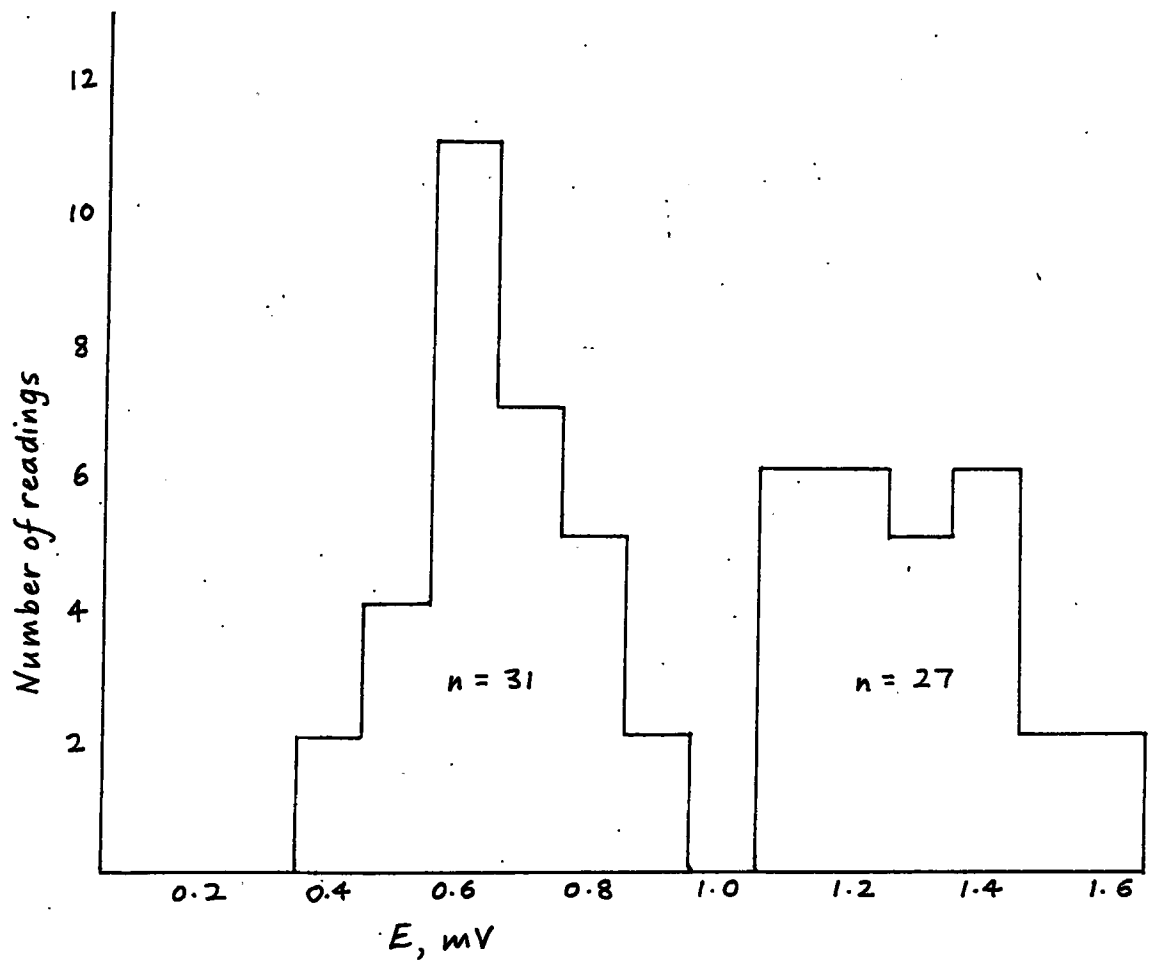


Figure 3.5: Histogram showing two peaks of readings in actomyosin, in A solution.

	I, mM:	140	105	69	29	Table:
Myosin		70	70	71	48	3.2
Myosin + C-protein		140				3.4
Myosin + C-protein + ATP		135				3.5
Myosin + ATP		106				3.7
Myosin + ADP		135				3.7
Myosin + ADP + DAPP		115				3.7
Myosin + DAPP		76				3.7
Phosphorylated myosin		115				3.9
Phosphorylated myosin after phosphatase		105				3.9
Phosphorylated myosin + C-protein		119				3.9
Phosphorylated myosin + C-protein after phosphatase		89				3.9
Myosin + 10mM PPi		65			66	3.10
Myosin rod		53	66	51	15	3.3

Table 3.21: Comparative myosin results, e/molecule.  
Errors are given in the relevant Tables.

I, mM :	210	140	105	69	29	Table:
AN concentrations, mean	9	8	8	8	6	3.11
Ultracentrifuged actin	9	9	9	8	6	3.12
Sonicated and rested actin			6	8		3.14-15
Stirred actin			8	11		3.14
Actin-tropomyosin-troponin	14	23		25	20	3.16
Actin + 1mM EGTA		13			10	3.17
Actin-tropomyosin-troponin + EGTA		19			26	3.17
Actin + 10mM PPi		8			5	3.10

Table 3.22 : Comparative actin and actin-tropomyosin-troponin results, e/monomer.

Errors are shown in the relevant Tables.



## CHAPTER 4

### THE STRUCTURE OF THE PROTEIN GELS

#### Section 4.1 : Light Microscopy of Gels

4.1.1 Insertion of microelectrodes into the protein gels was routinely carried out under a light microscope. While the principal reason for this was to facilitate accurate insertion, it also enabled qualitative observations to be made on the difference in appearance and in texture between the different protein gels. Observations were necessarily impressionistic, but nevertheless contributed to the overall picture of gel structure.

4.1.2 Myosin gels generally appeared very smooth and semi-transparent, very uniform in appearance and apparently uniformly packed. They were easy to measure, in that the microelectrode tip came cleanly out of the gel after insertion: the gel was not sticky. The addition of C-protein to myosin and to myosin rod produced a gel with perceptible iridescence.

4.1.3 Actin gels were usually almost transparent. At the lower concentrations, in which the effects of sonicating and stirring were observed, sonicated or rested gels were less opaque than stirred gels, but in neither state was the gel as transparent as the highly-concentrated ultracentrifuged gels.

4.1.4 Actomyosin appeared, by contrast, somewhat granular and porridgy in appearance, and quite opaque. This gel had a tendency to stick to the microelectrode tip, and also to clog it more frequently than did the other gels.

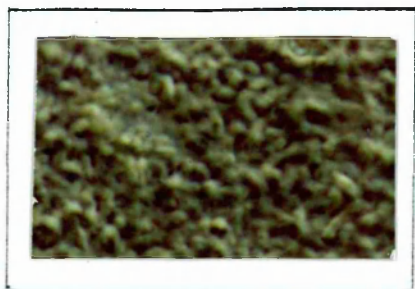


Fig. 4.1 : Myosin gel in A solution, 212 mg/ml

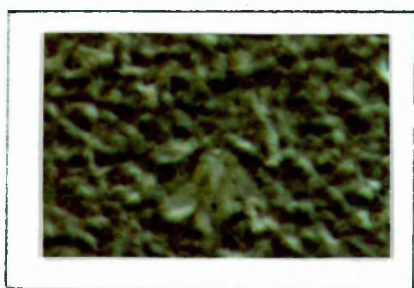


Fig. 4.2 : Myosin + ADP in A solution, 186 mg/ml



Fig. 4.3 : Myosin + ATP in A solution, 195 mg/ml

Figs. 4.1 - 4.3 : Ultracentrifuged myosin gels,  
magnification  $\times 1000$  : segments of  
Polaroid pictures

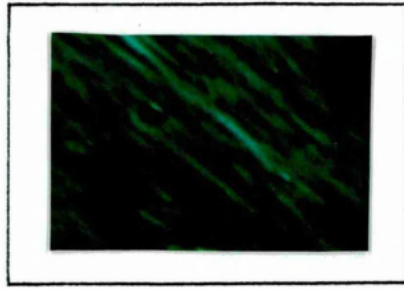


Fig. 4.4 : Ultracentrifuged actin gel in A solution

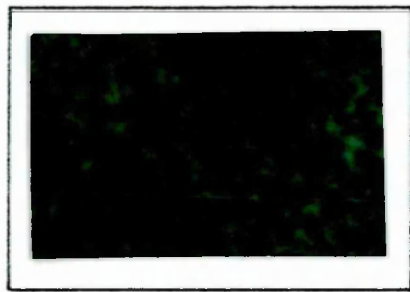


Fig. 4.5: actin gel, 65 mg/ml, stirred before photograph

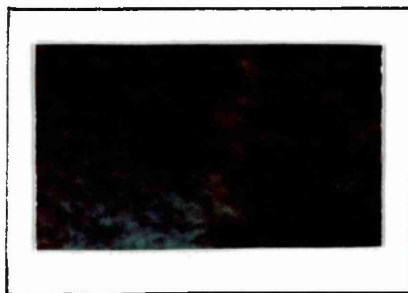


Fig. 4.6 : actin gel, 65 mg/ml, rested overnight

Figs. 4.4 - 4.6 : Actin gels, magnification X 1000:  
segments of Polaroid pictures

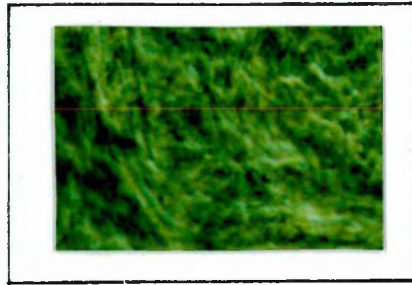


Fig. 4.7 : Actomyosin gel in A solution, 46 mg/ml; magnification X 1000 : segment of Polaroid picture

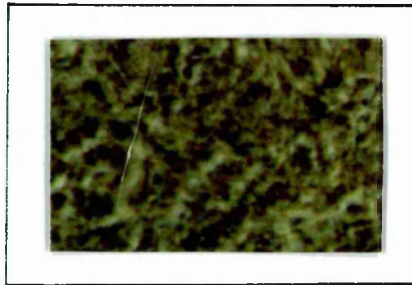


Fig 4.8 : Actomyosin gel, as above.

4.1.5 Samples of gels, in the 0.1mm sandwich cuvette used for spectrophotometric measurements of concentration, were photographed with a Polaroid camera under the light microscope. These are shown as Figs 4.1-4.8. It may be seen that, at this magnification ( $\times 1000$ ), myosin shows a regular pattern of aggregation (Fig 4.1). This does not appear to be significantly altered by the addition of ADP (Fig 4.2) or ATP (Fig 4.3), despite the differences these make to the fixed charge (section 3.1.7). In neither case does the myosin look more (or less) linearly ordered, or clumped, nor does the size of each clump (generally a sausage shape about 0.001mm in diameter) seem to vary. Myosin at this magnification does not suggest a link between charge and state of aggregation such as is found in actin (section 4.1.6). However, it remains possible that aspects of overall gel structure may have been disrupted by the process of compression between the plates of the sandwich cuvette.

4.1.6 Actin which was rested overnight (Fig 4.6), or which was ultracentrifuged (Fig 4.4), clearly shows ordered linear aggregation. By contrast the stirred actin (Fig 4.5) shows this as having been disrupted, with a less ordered structure resulting. Ultracentrifuged actin (Fig 4.4) is a firm gel, in which the filamentous structure might be expected to survive compression in the cuvette, as indeed it appears to have done. The low-concentration rested gel (Fig 4.6) is much looser, and it would not have been surprising had its linear aggregation been lost in the process of being scooped and flattened in the cuvette. Nonetheless linear arrays are still visible, though less marked than in the ultracentrifuged gel. The stirred gel (Fig 4.5) shows a few linear formations, but is principally made up of small grainy clumps or very short curled filaments. It is not likely that these are in reality longer filaments seen in transverse section, since the gel is loose enough for any filaments to align themselves horizontally (through a simple gravitational effect) when a small blob of gel is placed on a horizontal surface (in this case the bottom plate of the sandwich

cuvette). It is also unlikely that any linear formations were lost through disruption, since this did not occur in the case of the rested gel.

4.1.7 Actomyosin (Figs 4.7 and 4.8) appears considerably less ordered and less uniform than either myosin, or actin in any of its states. It shows a mixture of clumps and linear aggregations, apparently randomly distributed. It is not possible with confidence to determine whether, as might be supposed by inference from the preceding pictures, the linear filaments are actin and the clumps myosin, or whether this is a true actomyosin compound in which the two proteins are complexed together. However, the linear structures certainly appear thicker than those in the actin gels.

## **Section 4.2 : Electron microscopy of gels**

4.2.1 A number of gels were examined under the electron microscope: this was not the complete range of gels, and was undertaken principally to confirm that the appearance of gels of proteins studied in this way corresponded with that observed in other studies of aggregates of these proteins.

4.2.2 Small pieces of gel were fixed in 1% glutaraldehyde. They were then washed in the appropriate A solution and fixed in 4%  $\text{OsO}_4$ , 0.2M S-collidine for an hour. This was followed by a thorough washing in distilled water before staining with 2% uranyl acetate at 60°C. After further washing the samples were progressively dehydrated in graded ethanol. They were then impregnated with propylene oxide,



4.2.6 Ultracentrifuged myosin appears as ordered filaments (Figs 4.9 and 4.10). It is shown first in cross-section (Fig 4.9), where the filaments are seen as large dots and exhibit, in parts, classical hexagonal packing. In linear section (Fig 1.10), packing is seen as fairly uniform. There is a suggestion of cross-bridges on some of the filaments. The filaments appear to vary in length; however, it is not possible to tell whether this is, instead, merely an impression produced by the cutting angle.

4.2.7 Ultracentrifuged actin (Fig 4.11) appears as small filaments of varying lengths; these do not form regular arrays like the hexagonal packing found in myosin, but rather appear as a uniform network. The absence of any sections where the filaments are clearly either in cross-section or in linear arrays suggests a three-dimensional network. Within this, the actin fibres seem quite well aligned, and appear to have a uniform beaded structure.

4.2.8 In the low-concentration actin gels, sonicated and rested actin (Fig 4.12) is, like ultracentrifuged actin, uniformly networked; however, the filaments seem to be shorter and less variable in length. There is a suggestion of some directionality, although again nothing like a hexagonally packed structure. Filaments appear rather straighter than those in the ultracentrifuged gel. Unfortunately this is a particularly blurred picture; a further study was not possible within the time constraints.

4.2.9 Stirred low-concentration actin (Fig 4.13), while also clearly forming networks, is more disordered in appearance and seems to be collected in random tangles: the overall picture suggests fairly long filaments, tangled together.

embedded in epoxy resin and hardened overnight\*.

4.2.3 Sections of the embedded protein were made using a microtome and a glass knife. Copper EM grids were coated with 0.25% Formvar in ethylene dichloride, and protein sections were floated on to these. They were then stained with 2% uranyl acetate solution, washed, stained with lead citrate (Reynolds, 1963) and finally washed again.

4.2.4 A JEM-T7 electron microscope was used to examine the sections. This machine was used because it was the only one readily available, and it was deemed adequate for the present purpose. It had, however, a number of limitations consequent upon its considerable age: these included focussing difficulties and a lack of high resolution.

4.2.5 The observations should, of course, be treated with caution: the very small quantity of protein examined inevitably carries the possibility that the sample is unrepresentative, and the severity of the experimental procedures to which it has been subjected means that some disruption of the original state of the protein cannot be ruled out. Nevertheless, these observations accord well with other studies of these proteins, and tend to confirm observations made under the light microscope.

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\* These procedures were passed on by Dr Peter Cooke, now at the Electron Microscopy Center, USDA-ARS-EERC, 600E Mermaid Lane, Philadelphia, Pennsylvania 19118.



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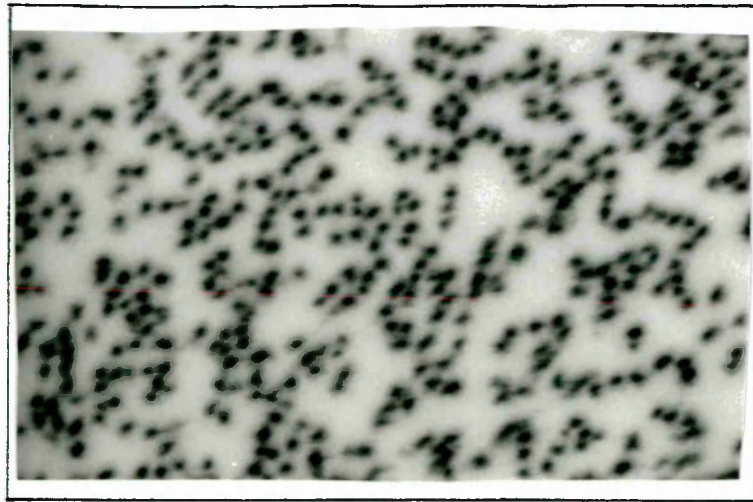


Fig. 4.9 : Ultracentrifuged myosin in A solution,  
X 240 000 : segment of micrograph,  
showing cross-section



Fig. 4.10 : Ultracentrifuged myosin in A solution,  
X 240 000 : segment of micrograph,  
showing linear section

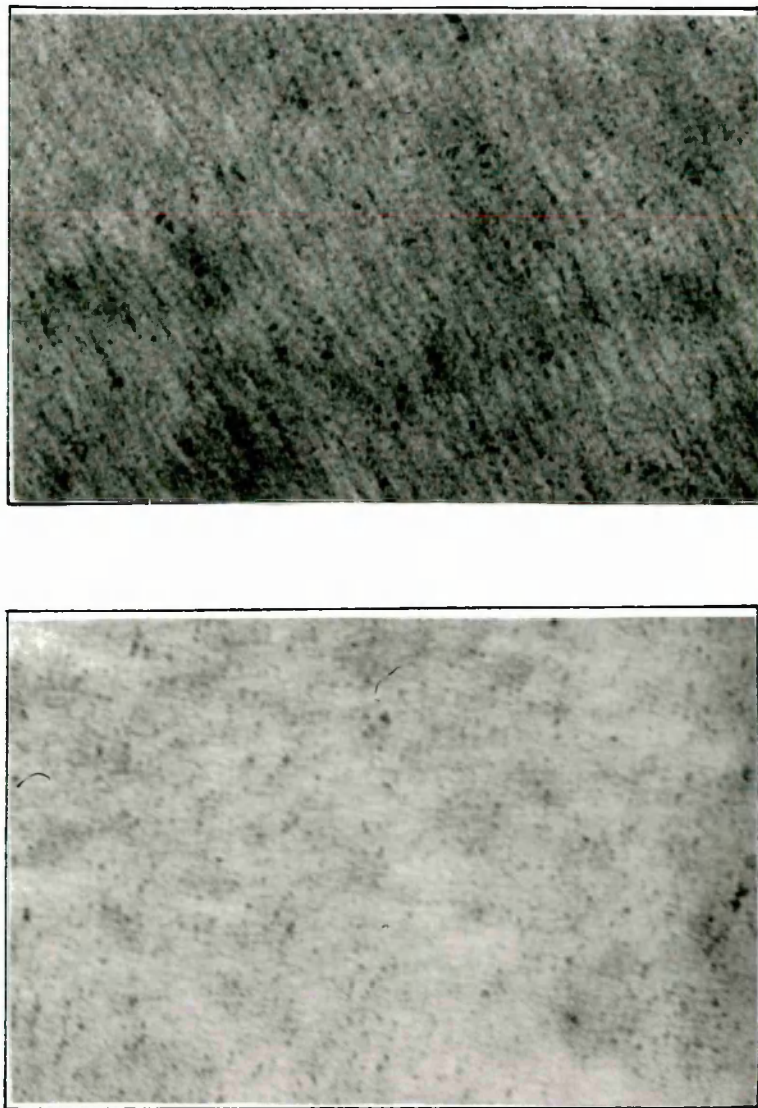


Fig. 4. 11 : Ultracentrifuged actin in A solution,  
X 240 000: segments of micrographs

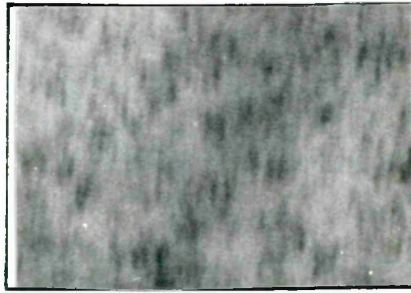


Fig. 4.12 : Actin gel, 55 mg/ml, rested,  
X 240 000 : segment of micrograph

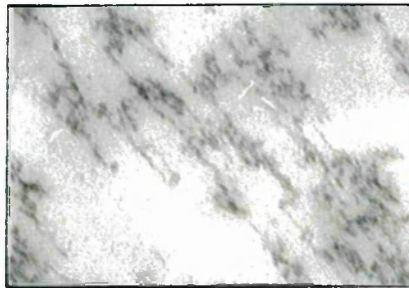


Fig. 4.13 : Actin gel, 55 mg/ml, stirred,  
X 240 000 : segment of micrograph

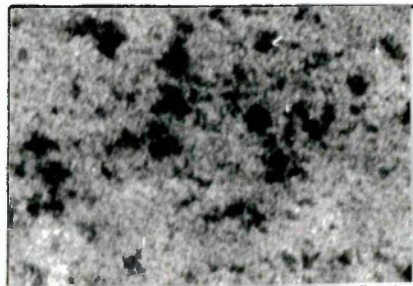


Fig. 4.14 : Ultracentrifuged actomyosin gel,  
X 240 000 : segment of micrograph



4.2.10 Actomyosin (Fig 4.14) appears disordered and messy, with deeply-stained dots or clumps. The probable interpretation is that these dots are myosin filaments, seen in cross-section. As in the myosin alone (Figs 4.9 and 4.10), apparent projections on some of the dots may be cross-bridges, but this cannot be assumed with confidence. The myosin dots are surrounded, it appears by a network of actin filaments\*. The beads of actin are clearly discernable, and the actin filaments are in most places close-packed.

There is no evidence of hexagonal packing of myosin in the actomyosin gel. This may be because any regular packing is hampered by the presence of the actin network, either as a result of simple steric hindrance, or because of the excess actin in the gel, or both. There is no suggestion of large discrete areas of single protein, either actin or myosin.

It is surprising that no myosin filaments in longitudinal section are observable. It is possible to conjecture that the lines of dots visible in parts of the picture are in fact broken, or wavy, filaments, but this is a very speculative interpretation and itself gives rise to further questions about the state of the actomyosin compound (see chapter 5).

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\* This gel contains excess actin.



## CHAPTER 5

### DISCUSSION

#### Section 5.1 : The Microelectrode Measurement of Donnan Potentials

5.1.1 The experiments described here were predicated upon the validity of the microelectrode technique for measuring the Donnan potential in a fixed polyelectrolyte system (Collins & Edwards 1971; Elliott 1973). A Donnan equilibrium or Donnan potential exists in a system in which the movement of ions in one direction, in obedience to an electrical gradient, is balanced by their movement in the other direction in obedience to a concentration gradient: thus at equilibrium an electrical potential exists, the magnitude of which is given by the Nernst equation. It is not dependent on the presence of a boundary membrane, but simply on the conjunction of two separate phases, one of which contains large ionized molecules which cannot move into the other phase.

In conventional Donnan experiments, sharp microelectrodes with an external tip diameter of  $0.5 - 1.0 \mu\text{m}$  are used. However, the microelectrodes used in the experiments described here had their tips broken (Section 2.4 above), giving them an external diameter of  $20-100 \mu\text{m}$ . Can a large-tipped microelectrode be expected to average in the way that a sharp microelectrode does (Elliott and Bartels 1982), or is it likely to introduce systematic artefacts?

5.1.2 Broken microelectrodes, used in these experiments, have very little resistance (less than  $2\text{M}\Omega$ ) - in fact, a check on the resistance of each microelectrode was a good indicator of its viability, since clogging generally causes a rise in resistance. However, sharp microelectrodes, used in standard microelectrode experiments in

skinned or glycerinated muscle fibres, and in threads of muscle protein (Cooke et al 1987), typically have a resistance of between 5 and 50 M $\Omega$ . Results from studies with sharp microelectrodes have been widely replicated and substantiated over the past ten years. In order to confirm that the lack of resistance on broken-tipped microelectrodes does not introduce an artefact, and that the results from gel experiments were comparable with those in intact muscle fibres, an experiment was arranged in which a 10 M $\Omega$  resistor was coupled into the circuit between the microelectrode holder and the meter. The readings obtained in this way exactly corresponded with those obtained in the same gel without a resistor.

5.1.3 With the sharp microelectrode it is important to avoid any artefact due to tip potential (Adrian 1956). A tip potential is a junction potential, which can be in the order of 10mV, arising from an electrical double-layer caused by surface charges on the sides of the microelectrode tip (Naylor 1977). Tip potentials do not occur on broken microelectrodes, as the sides of the tip are too far apart for this effect to arise.

5.1.4 A possible problem, which it is easy to envisage applying to large-tipped microelectrodes, is that of leakage or diffusion of 3M KCl from the microelectrode tip. Elliott & Bartels (1982) suggest that this does not apply to sharp microelectrodes, but it could be supposed to have an appreciable effect with tips up to ten times larger in (exterior) diameter. Empirically, however, measurements in gels suggest that this is not the case: if it were, repeated readings would be expected to show a downward trend, and the reading on a single insertion in which the microelectrode is left in the gel for a period of time (up to 60 seconds) would be expected to drift downward during this period; neither of these effects is observed.



5.1.5 Another possibility is that a large microelectrode tip might, because of its bulk, compress, disrupt or distort the gel matrix in such a way as to alter its charge in the vicinity of the tip. It is not easy to devise an experiment to demonstrate conclusively that this does not happen. However, the consistency of readings and of results, despite differences of up to 400% in the size of the tips, strongly suggests that this is not a factor.

5.1.6 It is unfortunate that it has not proved possible to measure gels with sharp microelectrodes (the tip immediately clogs, because of its small diameter) nor intact muscle fibres with broken microelectrodes (the tip is too blunt, and too large, to push its way into the fibre), so that no direct comparison of results can be made. However, the differences between the two sets of findings may be explained by a number of (complementary) factors. Indeed, these very differences offer some useful insights into the respective characteristics of the intact filaments and of their constituent proteins. This is discussed in more detail in Section 5.4.

5.1.7 Differences between the A-band and myosin, and the I-band and actin, mean that a precise correspondence between the charge characteristics of each is improbable. Intact muscle contains components other than actin, tropomyosin, troponin and myosin; the A-band contains the M-line proteins, and, importantly, C-protein, which binds strongly to myosin (Moos et al, 1975). My experiments (section 3.1.6) suggest that in the presence of C-protein, the myosin charge is substantially enhanced. The I-band includes a number of components, the removal of which affects the viscosity of actin (discussed below, Section 5.5.6). In addition, in intact muscle fibres (Bartels & Elliott, 1985), and in myosin threads (Cooke et al, 1987), the filaments are linearly oriented in a uniform direction throughout. All these factors are shown to affect the charge on the protein molecule.

## **Section 5.2 : Gels of two or more proteins: interpreting the C-protein results**

5.2.1 In order to calculate with confidence the charge per molecule in any mixture of two proteins, it is necessary to know in advance the charge on one of the two proteins and to make the assumption that this charge does not change. From this it is possible to calculate the charge on the second protein. If these conditions cannot be met, the best that can be arrived at is only the most likely of a number of alternative hypotheses.

5.2.2 Where the total protein charge is higher than predicted by the charge on the known protein, possibilities include the following: a net zero charge on unknown protein X and a change to the charge on known protein Y; a negative charge on X, added to the unchanged negative charge on Y; a positive charge on X and an increased negative charge on Y. The experiments with C-protein and myosin can only be interpreted in terms of more or less probable alternatives.

5.2.3 If, as seems likely from its amino-acid composition (Yamamoto & Moos, 1983), C-protein itself has a zero net charge, the addition of C-protein to myosin must in some way increase the myosin charge. If the Pr-/mM charge in col 1 of Table 3.4 is produced by only that 73% of the total protein which is the myosin, then the net charge per myosin molecule must be 131e.

5.2.4 In the intact thick filament, C-protein makes up about 2.5-3.5% of the thick filament mass (Harrington, 1979), so that in all myosin-C-protein gels there is C-protein very considerably in excess of that expected to produce an effect comparable to that in vivo. (At the stage of preparation, it had been assumed that C-protein would bind to myosin in a stoichiometric ratio, with the remainder being removed in

the supernatant.) In the experiment in which C-protein was combined with phosphorylated myosin, the C-protein in the gel was only 7%, but this is still well in excess. It is not clear why the proportion of C-protein retained in the gel should have varied to the extent that it did.

5.2.5 Since Table 3.4 shows that, if C-protein has a zero net charge, the myosin charge is higher in the gels with smaller percentages of C-protein, it is possible that the large excess of C-protein is preventing the maximum effect from being achieved, perhaps through steric hindrance. The observed myosin rod-C-protein charge (Table 3.6), where C-protein made up 43% of the protein gel, tends to support this possibility.

5.2.6 It is not likely that the C-protein molecule is itself directly contributing the additional negative charge, since on such a hypothesis 14% C-protein would be producing far more charge than 27% C-protein. In the absence of an increase in the myosin charge, it would also imply an improbably high negative charge on C-protein of between 300e and 600e per molecule. The amino-acid composition of C-protein appears to rule this out completely (Yamamoto & Moos, 1983).

5.2.7 The third possibility is that C-protein has a positive charge. This is given some credence by the observation that gels with small percentages of C-protein showed higher charges. If the net charge on either molecule does not vary, this hypothesis suggests a myosin charge of approaching 200e/mol or more, offset by a positive charge on C-protein of about 65e/mol. Again, however, the amino-acid composition makes this most improbable.

5.2.8 The addition of ATP to myosin-C-protein does not increase the charge above its already elevated level. Harrington (1979) reports that C-protein has an appreciable effect in depressing the ATPase activity of in vitro actomyosin systems. Moos & Feng (1980) confirmed that this was the case for muscle myosin at low ionic strengths, but found that at ionic strengths above about 125mM it produced, instead, an activating effect: this would apply in the case of gels in A solution where the ionic strength is 140mM. However, in the absence of actin it is not clear that ATPase activity would take place.

### **Section 5.3 : Ionic strength effects in gels and in intact fibres**

5.3.1 A clear ionic strength dependence is observed in both myosin and actin gels (see Table 3.1 and Fig 3.2).

5.3.2 In myosin the charge is highest at  $I=69\text{mM}$ , but this is only marginally higher than the charge at  $I=140\text{mM}$ , which approximates to the ionic strength under physiological conditions. The charge falls quite sharply between  $I=69\text{mM}$  and  $I=29\text{mM}$ . In effect, therefore, a constant charge level is maintained from physiological ionic strength down to about half physiological ionic strength, and falls to two-thirds of this value at one-fifth physiological ionic strength.

The charge in the actin gel is highest at physiological ionic strength,  $I=140\text{mM}$ . It falls slightly at  $I=105\text{mM}$ , but does not fall again until below  $I=69\text{mM}$ .

5.3.3 Naylor et al (1985), and Bartels and Elliott (1985), using glycerinated rabbit psoas muscle, find that in rigor solutions (identical to A solutions) the thick filament charge also decreases as ionic strength falls. This is the linear charge per

nanometer, and X-ray diffraction measurements have been used to correct for changes in volume as a function of ionic strength. In A solution Naylor et al found a charge of 47e/nm, falling to 40e/nm in 0.2A and to 22e/nm in 0.05A. Bartels and Elliott, using chemically skinned rat muscle, found an A-band charge of 51e/nm in A solution, falling to 43e in 0.2A and 38 in 0.1A.

5.3.4 Over the ionic strength range measured in gels, the profile of the curve is similar, supporting the probability that the myosin in the A-band is a major contributor to the charge characteristics observed in the A-band.

5.3.5 Both Bartels & Elliott and Naylor et al conclude that the thin filament charge is less dependent on ionic strength than is the thick filament. Gel results suggest that there is an ionic strength effect, though rather less marked than that found in the A band or in myosin, but that this effect may be missed because the absolute values concerned are low (the difference between 7e and 8e may appear negligible by comparison with the difference between 70e and 80e, although in percentage terms the differences are identical).

## **Section 5.4 : Differences between the charge in the gel and that in intact fibres**

5.4.1 There is a discrepancy in the values (as opposed to the ionic strength dependence) of the charge recorded in gels and in intact fibres, particularly apparent in the case of myosin. Myosin gel at physiological ionic strength gives a charge of  $70 \pm 3$  (se) e/molecule, whereas the A-band of glycerinated rabbit muscle in the same solution shows a charge in the order of  $250 \pm 60$  (ee) e per molecule, derived from the linear charge per nanometer in Naylor et al 1985\*. See Table 5.1. A much smaller discrepancy exists between gels of actin-tropomyosin-troponin, which at physiological ionic strength gave a charge of  $23 \pm 12$  (ee) e/monomer, and the I-band charge of approximately 36e/monomer derived from Bartels & Elliott 1985\*\*.

5.4.2 Sequence data for myosin (McLachan and Karn, 1982; Grand, 1982 and Karn et al, 1983), for actin (Collins and Elzinga, 1975; Vanderkerckhove and Weber, 1978), tropomyosin (Stone and Smillie, 1978) and the troponins (Collins et al, 1977; Pearlstone et al, 1977; and Wilkinson and Grand, 1975), allow the theoretical charge on each molecule to be calculated (Naylor et al, 1985).

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\* Calculated on the basis that a thick filament 1.6mm long contains 300 molecules (Kensler & Stewart, 1983), giving 0.19 molecules per nanometer.

\*\* Calculated on the basis of an actin subunit repeat of  $54.6\text{\AA}$  (Harrington 1979), giving, for a double helix, 0.37 molecules per nanometer.

Ultracentrifuged gel:		
actin-tropomyosin-troponin	23 ± 12 (ee)	
myosin	70 ± 3 (se)	
Glycerinated muscle fibre:		
I-band	36	
A-band	220-260	
Amino acid prediction	16-26	46-128

Table 5.1 : Comparison of charge (e/monomer) in protein gels and in intact fibres in A solution. I-band figure from Naylor et al 1985; A-band figure from Bartels & Elliott 1985.

5.4.3 The charge for actin which Collins & Elzinga give in their paper is 7e per molecule, which accords with the charge of 6-9e found in the actin gel. The thin filament contains tropomyosin and the troponin molecules in addition to actin, so there is not a direct correlation between the two. On the basis of the sequence data the total thin filament charge is approximately 5.4e per nanometer, compared with Naylor et al's figure for the thin filament of glycerinated rabbit muscle of 6.5e in A solution. It is puzzling, however, that the latter rises as ionic strength falls, reaching a peak of 15 at  $I=29\text{mM}$ .

5.4.4 The calculation from actin sequence data of 7e assumes that all 8 histidines are ionized, and this is the tacit assumption made by Collins & Elzinga. However, the pK of histidine is 6.5, and at pH 7.0 it might be more reasonable to assume that only half the histidines are ionized, which would give a charge of 11e, or a range of 7-15e\*.

On this basis the combined troponins give a range of 11-22e and tropomyosin 50-54e\*\*. The range of charge per monomer is 15.7 - 25.8e, and the thin filament charge per nanometer could therefore be predicted to be in the range 5.8e/nm - 9.6e/nm, calculated on the basis that one actin molecule is 2.73nm long and that

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\* If the net charge on actin includes bound calcium, the negative charge per monomer falls by 2 electrons per monomer.

\*\* Tropomyosin: each of the two chains consists of 80 negative groups, and 55 positive groups including two histidines. Of the troponins, troponin T has 61 negative groups, and 70 positive ones including six histidines; troponin C has 45 negative groups, and 16 positive ones including one histidine; and troponin I has 35 negative groups, and 44 positive ones including four histidines.



tropomyosin-troponin associates with actin in the ratio 1:7. This in fact accords better with the measurements by Naylor et al than the figure of 5.4e/nm advanced in their paper. Therefore, at physiological ionic strength and at 2 x dilution ( $I=71\text{mM}$ ), Naylor's figures agree with the charge found in the actin and in the actin-tropomyosin-troponin gels.

5.4.5 Converting Naylor et al's charge, expressed in e/nm, into e/monomer, on the basis of a monomer consisting of one actin molecule plus one-seventh of a tropomyosin-troponin complex, and on the basis of a 2.73nm repeat for a single actin subunit (ie half of a double strand with a subunit repeat of 54.6Å), gives a figure of 17.6e/monomer in A solution ( $I=140\text{mM}$ ) and 20 e/monomer at 0.5A ( $I=71\text{mM}$ ). This compares well with the charge found in gels of actin-tropomyosin-troponin, where a mean charge of  $23 \pm 12$  (ee) e/monomer was found in A solution and a charge of 25e/monomer in 0.5A. In 0.2A solution the gel charge was  $20 \pm 6$  e/monomer, in contrast to Naylor et al's figure of 40.5e, however.

5.4.6 In 0.2A gel experiments, less tropomyosin-troponin than expected was retained in the final protein gel, and although the calculation of the charge per monomer made allowance for this it is possible that the charge was nonetheless affected. On the other hand the 100% increase in I-band charge, in Naylor et al's experiments, from 0.5A to 0.2A, is surprising, especially considering that the charge in the A band has been found to fall over this ionic strength range and that in the I band has on the whole been observed to remain constant or, if anything, also to fall. (Naylor et al used a very indirect method for measurement of the I-band charge, and they remark in their paper on the high level of uncertainty that resulted). It is not possible to determine whether one of these figures is likely to be more accurate than the other: whether, on the one hand, the figures at low ionic strength are

anomalous and the I-band charge and the gel charge are essentially showing the same picture; or whether, on the other hand, the lower figures at the higher (and physiological) ionic strengths are the anomalous ones and the I-band charge should be seen as generally higher than the gel charge. This latter interpretation would perhaps accord better with the more direct measurements in glycerinated rabbit muscle by Bartels & Elliott, who found the equivalent of 35.5 e/monomer (calculated from their e/nm figures) at ionic strengths from 140 mM (A solution) to 30mM (0.2A), falling to 24.6 e/monomer only in 0.1A, for which there is no gel comparison.

5.4.7 It is conceivable that the level of charge found by Bartels & Elliott in the I band is artificially high as a result of the method of measurement. It relies on the exceedingly accurate impalement of the I-band section of a striated muscle fibre with a sharp microelectrode. This is done under a light microscope but is nonetheless not an easy procedure, and the small size of the I-band is such that the possibility of impaling the Z-line instead cannot be excluded (G F Elliott, personal communication). This could give a considerably higher charge than the I-band itself, since it contains an array of proteins including  $\alpha$ -actinins and capping proteins; the N-line, close to the Z-line, contains nebulin. All these could give false I-band readings which are higher than true I-band impalements, and thus raise the mean charge figure artificially. However, clearly it is not possible to quantify such an effect.

5.4.8 If, on the other hand, sequence data predictions, gel charge measurements and the measurements of charge in intact fibres are all giving an accurate picture and the charge on the proteins concerned therefore varies or changes, it is likely that this is the result of changes in the ability of all or some of the proteins to bind ions.



5.4.9 This could be due to shifts in the conformation of individual molecules, such that binding sites are exposed or occluded; it could be due to co-operative effects between molecules or filaments in an array; it could be due to co-operative effects induced by complexing two or more proteins; or it could be due to dynamic effects within the filament itself or between the thin and thick filaments in the intact fibre. It seems likely that a combination of these effects operates, and it would not therefore be a priori surprising to find a higher (or at least different) charge in the intact fibre than in preparations of gels of its constituent proteins.

5.4.10 One very obvious possible factor is the degree of order to be found in the different preparations. Even if the gel contained identical proportions of all the constituents of the intact fibre, they would not be assembled and aligned in precisely the same way; and the arrangement of the proteins relative to each other is likely to be a major factor in ion binding. Egelman (1985) reports that order is imposed on actin by myosin binding (as in muscle fibres in rigor), perhaps by bound S-1 inhibiting the ability of actin subunits to rotate. The effect, on actin filaments decorated with S-1, is a six-fold increase in helical order. This does not, however, appear to be reflected in the charge characteristics of the gel (see discussion of actin-S-1 gel, section 5.7). Further factors potentially affecting actin charge are discussed in section 5.5.

5.4.11 The very much larger discrepancy between the myosin gel charge and the charges in the A band reported by Bartels and Elliott and by Naylor et al raises further questions. The calculated charge from the sequence data for (nematode) myosin (McLachan and Karn 1982; Karn et al 1983) gives a net charge of 90e, with the small positive charge of 2+ on each myosin head offsetting the negative charge on the rod portion of 94e. Each myosin head has two light chains associated with it,

adding 19e to each head, giving a total myosin charge of up to 128e. There are 34 histidines on the rod section, 21 on each head and 3 on the light chains attached to each head; if all were ionized, the charge would fall to 46e. The charge found in the myosin gel, 48-70e depending on ionic strength, accords with this range, and at physiological ionic strength and pH the gel charge of 70e suggests that about 70% of the histidines are ionized. At all events, both sequence data and gel observations are very much lower than the 220-260e/molecule found in the intact fibre in A solution (rigor solution).

5.4.12 Again, one factor is the presence in the A-band of various proteins other than myosin, notably C-protein. C-protein may contribute charge of its own in addition to the more likely circumstance that it enhances the charge on myosin (see section 5.2). In gel experiments, C-protein appeared to increase the myosin charge by about 100%, although these gels contained C-protein in excess of that found in intact fibres. It is indeed conceivable that lower levels of C-protein might produce an even higher myosin charge, since extra charge produced was in inverse relationship to the amount of C-protein retained in the gel (section 3.1.6 and section 5.2.3 - 8). Koretz (1982) states that myosin aggregation behaviour is affected by C-protein: if C-protein is present in the myosin solution before aggregation, it appears to be incorporated in the filament backbone, and at high ratios (in the order of myosin:C-protein::5:1) alters or disrupts the filament structure (Moos et al, 1975).

5.4.13 Bartels et al (in preparation, 1991) measured the charge in threads of myosin and myosin rod extruded from a syringe. They report a charge of about 100 e/monomer in myosin in A solution, rising to 110 e/m 0.5A and falling to 65 e/m in 0.2A. These threads are considerably more ordered and aligned than myosin in the gel, as a result of the extrusion process. (Cooke et al 1987), and might therefore be

expected, for the reasons advanced in relation to actin (above), to show a higher charge due to greater ion binding capacity. Similarly, Bartels et al suggest that differences in packing between the thread and the A band may account for the difference in charge between the two, with the A band more than twice as charged. Tumminia et al (1989) show structural and morphological differences between native thick filaments and synthetic thick filaments of rabbit myosin, which are consistent with this hypothesis.

5.4.14 In the intact, glycerinated fibre the addition of ATP to the solution (relaxing solution) causes the A band charge to fall (Bartels & Elliott 1985). In the myosin gel the addition of ATP produces the opposite effect, giving an increase of 51% at physiological ionic strength, to a mean of  $106e \pm 10$  (sd) (Table 3.5). The binding of ATP to myosin molecules would not produce an effect of this magnitude by the simple addition of the charge on ATP (about 2 e/molecule). Similarly the slight increase in ionic strength caused by adding ATP to A solution would be expected to contribute to the charge only marginally, if at all.

5.4.15 The addition of ATP to a gel of myosin and C-protein gave a charge of  $135 \pm 30$  (ee) e/molecule, slightly lower than the charge found in myosin-C-protein alone and consistent with the A band charge on glycerinated rabbit muscle in relaxing solution, calculated from Bartels & Elliott's data to be 121 e/molecule where  $I=180$  mM and 159 e/molecule where  $I=86$  mM.

5.4.16 The addition of ADP to the myosin gel alone produces an increase in charge of 93%, also taking it to  $135 \pm 13$  (sd) e/molecule in A solution (Table 3.7). Unfortunately the companion experiment of adding ADP to myosin + C-protein gel was not undertaken, since its potential significance was not appreciated until after

	Pr <sup>-</sup> /mM	e/mol myosin	% C-protein	n
Myosin	15	70 ± 3 (se)	-	14
Myosin + C-protein	20	131 ± 20 (ee)	27	1
Myosin + C- protein + ATP	20	135 ± 21 (ee)	29	1
Myosin + ATP	22	106 ± 10 (sd)	-	5

Table 3.5 : Effect on myosin charge of the presence of ATP  
(from p. 48) and of C-protein, in A solution, I = 140

	e/mol	n
Myosin + ATP	106 ± 10 (sd)	5
Myosin + ADP	135 ± 13 (sd)	3
Myosin + ADP + DAPP	115 ± 25 (ee)	2
Myosin + DAPP	76 ± 14 (ee)	1
Myosin (control)	70 ± 3 (se)	14

Table 3.7 : Myosin charge in the presence of ATP; ADP;  
(from p. 49) and DAPP, in A solution, I = 140 mM.

the experimental series had been completed. It is possible, however, to envisage a model in which myosin-C-protein + ADP corresponds to the position in the intact A band in rigor, with its much higher charge, by analogy with the effect produced in myosin alone where the addition of ADP gives a much higher charge than the addition of ATP. This would suggest that a myosin-C-protein ADP gel might have a charge comparable to the approximate 250 e/monomer found in rigor A-band. On this basis it could be supposed that C-protein has an ordering or regulating effect on myosin and facilitates the effect produced by the nucleotide.

5.4.17 It is, however, still puzzling that nucleotide also produces a much increased charge in the absence of C-protein. Perhaps the binding of ADP or ATP to the myosin molecule entails a conformational change in some part of the molecule such that its ion-binding propensity is enhanced. Bartels et al (1991) found in experiments with myosin and with myosin rod that the charge change takes place in the rod portion of the molecule in the absence of the myosin head, despite the fact that the primary ATP binding site is on the head. This is, however, compatible with the finding, in the gel, that the charge on the myosin rod is a high proportion of the charge on the whole molecule, in accord with the charge predicted from sequence data. Bartels et al found, further, that ATP produced no charge change on LMM, concluding that the initiation site must be on the S2 subfragment. The important point would seem to be that the effect is not caused by nucleotide binding to the myosin head or, therefore, by any conformational changes transmitted by the head to the rest of the molecule. It is possible that a charge change of this magnitude could be a significant component of the mechanism of muscle contraction.

5.4.18 If charge changes are the result of ion-binding, an obvious possible candidate for the role of bound ion is phosphate. In an experiment in which the level of



phosphate in the solution was increased, and in the absence of chloride and magnesium (Table 3.8), the myosin gel showed a charge of 118 e/molecule at an ionic strength comparable to that of A solution, and of 104 e/molecule at an ionic strength comparable to that of 0.2A. Thus in the absence of all chloride, it seems likely that phosphate may be binding to sites which might otherwise be occupied by chloride. This is supported by the finding that when a small quantity of chloride is included in the high-phosphate solution, by the addition of 5mM MgCl<sub>2</sub>, the charge in A equivalent is 85 e/molecule and in 0.2A equivalent is 60 e/molecule, in each case somewhat raised from the control levels of 70 and 48 e/molecule respectively, but falling short of the charge in the complete absence of chloride, suggesting that chloride competes with phosphate for these binding sites. The alternative possibility here is that it is the magnesium which is the active ingredient; but it is less easy to envisage a model to explain the result on this basis. Such a model would seem to rely on magnesium having the capacity in some way to modify ion-binding.

5.4.19 Elliott et al (1985) suggest a mechanism for ion-binding, derived from a model put forward by Loeb and Saroff (1964) for chloride binding to ribonuclease. According to this model, anions are hydrogen-bonded to clusters of charged side chains. Loeb and Saroff, studying ribonuclease, suggest a cluster of three positive charges (protonated amino groups) with two carboxyl groups close enough to form hydrogen bonds: the chloride competes with the carboxylate ions to form hydrogen-bonds with the three amino groups and one carboxyl group in a tetrahedral array.

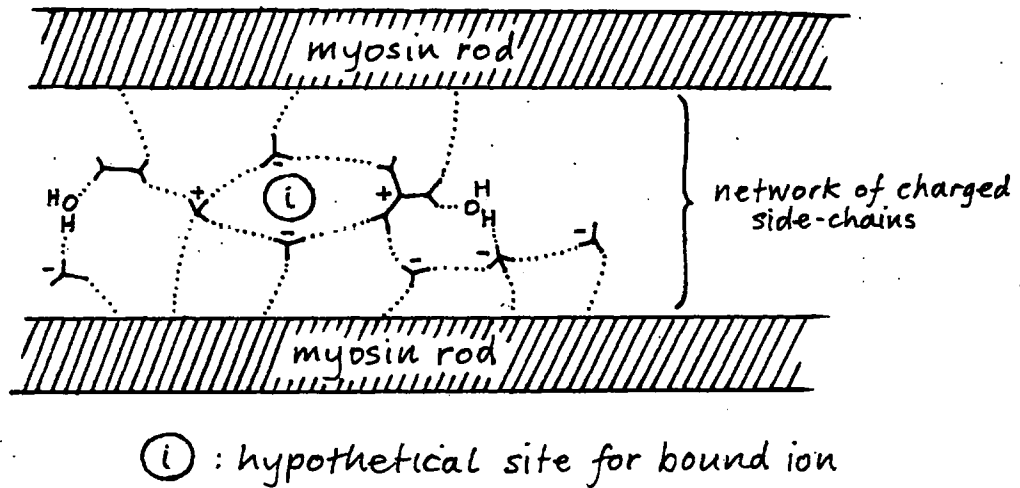


Fig. 5.1 : Schematic diagram of hypothetical Saroff site, not to scale. After Elliott et al, 1985.

5.4.20 Elliott et al propose that similar Saroff sites occur in the myosin filament, either between the myosin rods of different myosin molecules or between the two polypeptide chains of a single myosin rod (Figure 5.1). These sites might bind phosphate or chloride competitively, which is consistent with the results in the gel. It is suggested that the binding of ATP to a (secondary) ATP-binding site somewhere on the rod portion of the myosin molecule initiates the reduction in charge found in intact fibres and in myosin threads.

5.4.21 If this hypothetical mechanism were also involved in the charge changes in myosin gels, the more probable location of the Saroff sites would be between the polypeptide chains of the myosin rod, since it seems unlikely that the filaments of myosin molecules in the gel are as ordered and uniform as they are in the intact fibre, and this lack of order would, it can be supposed, tend to prevent the relevant side chains arriving at the appropriate relationship to each other. The structure of the myosin molecule itself is the same in either case, however, so sites on the rod would be conserved.

5.4.22 On the other hand it could be argued that the generally lower charge found in the gel might be the result of a lower number of Saroff sites in the gel than in the intact fibre, as a consequence of the potentially less ordered, or at least different, structure of the filaments (Koretz 1982).

5.4.23 However, experiments with gels of phosphorylated myosin and phosphorylated myosin plus C-protein also showed a high charge, 115 e/molecule in myosin in A solution and 119 e/molecule in myosin plus C-protein in A solution. In this case, since the agent was the enzyme myosin light chain kinase (MLCK), any

additional charge as the result of phosphate binding through phosphorylation is either on the myosin head or is mediated by the myosin head.

5.4.24 In these experiments, unlike others with myosin plus C-protein, the charge in the myosin-C-protein gel was not significantly higher than the charge in the myosin gel. This may be because phosphorylation of the myosin took place before the addition of C-protein; this would suggest that the C-protein itself has had no effect on the charge, which is in each case produced solely as the result of phosphorylation. Linked with this is the observation that the final myosin-C-protein gel was only 7% C-protein, as opposed to between 14% and 27% in the plain myosin-C-protein series in A solution. It could be conjectured that changes in the myosin molecule, brought about as a result of the action of MLCK, have reduced the ability of myosin to bind C-protein, and hence diminished or eliminated the charge-enhancing effect of C-protein binding.

5.4.25 The addition of 10 mM pyrophosphate (PPi) to A solutions appears to reduce the myosin gel charge in the nearest approximation to A solution, and to increase it in the approximation to 0.2A solution. Elliott et al (1985) found a charge decrease in myosin threads and in intact fibres on the addition of PPi; in the intact fibre this was comparable to the effect observed with ATP, and it would be reasonable to suppose that PPi is acting as an analogue of ATP. However, ATP dramatically increased the gel charge (as did ADP) whereas PPi does not. In some way, therefore, it appears that in the gel the presence or absence of the nucleotide is crucial.

5.4.26 It would, on the other hand, also be possible to explain the gel results as the simple operation of ionic strength and pH effects, since both these parameters

change with the addition of PPI. In A solution the effect is to raise the ionic strength from 140 mM to 174 mM and to lower the pH from 7.0 to 6.2. Since a decrease in pH generally results in a decrease in charge (Naylor 1977), and since an increase in ionic strength past an ionic strength of 140mM does not appear to produce a marked increase in charge (see section 3.1.10), it might be expected that the net effect would be downwards. In 0.2A the pH fell from 7.0 to 5.6; the ionic strength was raised from 29 to 64, and over this range a noticeable charge increase would be expected. The ionic strength of 0.5A solution is 69mM, with an mean charge of 71 e/molecule. Bearing this in mind, the mean charge of 66 e/molecule in 0.2A + PPI might be the expected net result of its upward tendency from the ionic strength and the downward tendency from the pH.

### **Section 5.5 : Actin: the effect of stirring the gel**

5.5.1 A completely unexpected finding of these experiments was the increased actin charge, in low-concentration gels, on stirring but not on sonication. The discovery of the stirring effect was made quite by chance, then replicated several times with identical results: a single sample of gel would show a high charge shortly after stirring, decline to the lower charge after remaining undisturbed for two to three hours, and could then be returned to the more highly-charged state upon re-stirring, a cycle which would only be limited by the life of the preparation.

5.5.2 Gel samples were sonicated in the expectation that this too would result in a higher charge, with sonication acting as a more extreme form of stirring. It was another surprise, therefore, when sonicated gels were found to have exactly the same, low, charge as gels allowed to rest for a period of hours or overnight. This effect was also replicated in a number of experiments and was quite clear.

5.5.3 The most likely explanation of the latter finding lies in the fact that sonicated gels have been found to re-polymerize very quickly and completely, and thus return to a state indistinguishable from that found in gels which have polymerized in the normal way over a period of time. Asakura et al (1963) report that in the presence of  $MgCl$  sonication of a cross-linked F-actin gel produces short chains of F-actin which re-polymerise in a few seconds.

5.5.4 Asakura et al's proposed models of F-actin substructure are reproduced in Figure 5.2, below.

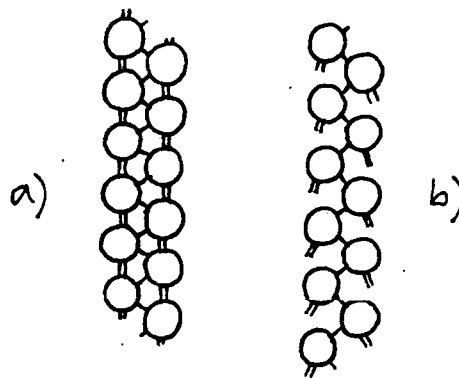


Fig 5.2 Models of F-actin substructure, after Asakura et al 1963: a) Intact filament, b) interrupted filament during sonication.

According to this model, actin during sonication is in a transitional state: in this state it is less viscous than normal F-actin, both because the filaments are shorter and because they are less rigid due to the partial interruption of bonds within the filament, between the adjacent monomers. After sonication ceases, this lowered viscosity, combined with regular dispersal, accelerates re-polymerisation.

5.5.5 It is not to be expected that stirring or syringing would interrupt the polymer structure in the same way. Rather than breaking bonds between monomers in the filament, a possibility is that stirring disrupts the much weaker (van der Waals) bonds between different filaments in the gel matrix, and tangles the filaments up sufficiently for steric hindrance to inhibit their subsequent re-linking, which then takes place only gradually. These three possible states are shown in diagrammatic form in Figure 5.3.

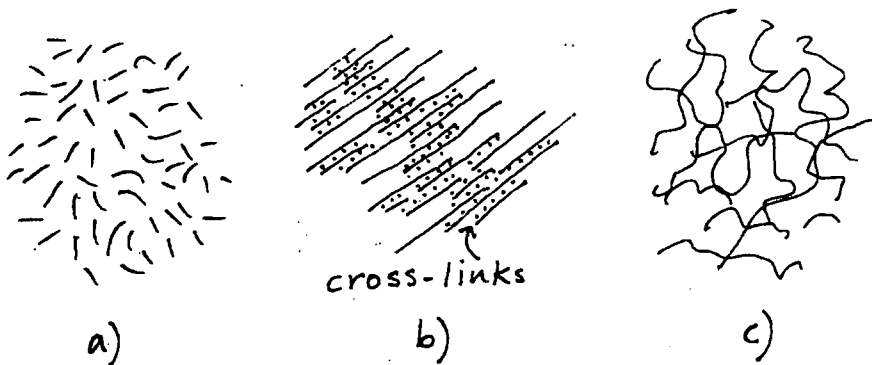


Fig 5.3 Diagrammatic representations of possible conformations of :

- a) actin during sonication
- b) polymerized, cross-linked actin after resting or after sonication
- c) stirred or syringed actin

In this model, it is possible that positive charges on the surface of the stirred filaments, which would normally be cross-linking into a gel matrix (as in diagram b), are unable to link to others and are exposed, and bind free negative ions such as phosphate. (These are not the hypothetical Saroff sites as in myosin, since Saroff

sites are likely to be the result of a considerable degree of order within the filament or gel).

5.5.6 On the other hand, if the rapid re-polymerisation of sonicated F-actin is primarily a factor simply of the very large number of very short filament fragments produced, and not of the partial interruption shown in Fig 5.2b, then perhaps stirring also produces partially interrupted, lengthened, but unbroken F-actin filaments. The breaking of the bonds shown diagrammatically in Fig 5.2 represent a shift from the preferred helical form to a stretched linear form. Asakura et al suggest that this process also liberates ADP molecules. If ATP molecules are present they will rapidly replace the ADP and the filament will quickly return to its original, shorter, helical conformation as ATP splitting is accompanied by the re-formation of the interrupted bonds. In the absence of ATP, however (as in the standard series of A solutions), the bond re-formation will occur very much more slowly, over a similar time-course to the observed change in charge from a stirred to a rested gel.

5.5.7 If this is a possible model for the stirred gel, what is causing the increase in charge? An obvious candidate must be the ADP, and it is suggestive that ADP was also implicated in a very considerable charge increase in myosin (Sections 3.1.7 and 5.4.16). However, it is not clear why the ADP charges are not being cancelled out by whatever positive charges, now free, had presumably masked them; unless these are now binding other negative ions.

5.5.8 Alternatively, stirring might be providing the system with sufficient energy to reverse, temporarily, the enzyme action by which the third phosphate ion is released after the splitting of ATP, and the higher charge is the result of the re-binding of



phosphate. Such a model would also accord with the recent observation that G-actin has a lower charge than (newly-polymerized) F-actin (Deshayes et al, 1991, and C. Deshayes, personal communication). This postulates a series of charge changes accompanying the stages in actin polymerisation. As G-actin splits Mg-ATP the magnesium ion is released: as this is a positive, divalent ion the (negative) charge increases. As polymerization takes place, this is followed in due course by the release of the phosphate ion; as this is a negative ion the charge, upon its release, goes down. Sonication would be assumed to accelerate this release, and stirring to reverse it.

5.5.9 Griffith and Pollard (1982), in a discussion of cross-linking in actin gels, report that the removal of minor contaminants by gel filtration greatly increases the viscosity of the gel: in other words, increases the cross-linking. Since the experiments with stirred and with sonicated actin gels indicate that a higher degree of cross-linking is associated with a lower charge, it might be expected that gel-filtered actin would show a lower charge than non-filtered actin, and equally that actin in the intact I-band, with nothing removed, might be expected to show a higher charge than extracted and purified actin in gel form.

5.5.10 It is conceivable that, when stirred, actin, rather than binding negative ions, releases positive ions, leaving its total net charge more negative. A possible candidate for release might be calcium, and if released stoichiometrically this would indeed subtract about two positive charges from each actin molecule, thus increasing the negative charge by the amount observed.

5.5.11 This possibility is consistent with the charge found in actin and in actin-tropomyosin-troponin gels in the presence of 1mM EGTA, which would be expected

to chelate bound calcium. In A solution in the presence of EGTA, the actin charge is 3e higher than the control without EGTA, and the actin-tropomyosin-troponin charge 4e higher than the control; in 0.2A, the difference is 4e and 5e. Within experimental error this suggests an effect similar to that found in the stirred gel.

5.5.12 The addition of 0.1mM  $\text{CaCl}_2$  to actin and to actin-tropomyosin-troponin gels, on the other hand, has no effect at all. This suggests that calcium binds only at the specific single calcium-binding site, and that that site is either already occupied, or is occluded and inaccessible. The former accords with the presumed chelating effect of EGTA.

### **Section 5.6 : Actin: the emerging picture**

5.6.1 Considerable research has been done into the mechanism and course of polymerization of actin. In nonmuscle cells the continuing processes of polymerization, depolymerization and repolymerization are likely to have specific functions (Korn et al, 1987). In muscle, however, polymerization is important only to provide and maintain the thin filaments required for contractile activity. Actin in the thin filament is all, or almost all, in a stable polymerized state, the classic double-stranded helix (Hanson and Lowy, 1963). Thus studies of the parameters affecting polymerization are not necessarily directly relevant to the functions actin may be performing in the muscle cell and to the role of actin in muscle contraction.

5.6.2 Polymerization studies have, however, found that the hydrolysis of ATP is independent of polymerization, and this may have implications both for the observed changes in charge and for the role of actin in intact muscle. Korn et al (1987) report that hydrolysis of ATP takes place on the F-actin filament after

polymerization, and that it follows two separate and distinct steps: cleavage of ATP results in a highly stable filament with bound ADP-Pi; inorganic phosphate (Pi) is subsequently released more slowly. Pi release may therefore have the potential to act as a regulator; for instance, as Korn et al say in conclusion, "It would be interesting to investigate whether the ability of Pi to cause muscle to relax might be due to the binding of Pi to the actin thin filaments rather than or in addition to binding to the myosin thick filaments." This possibility is certainly consistent with the charge changes found in actin in the present work.

5.6.3 Recent work by Carlier and Pantaloni (1988) also suggests a role for inorganic phosphate as an actin regulator. They show that there is an exchange of bound Pi on F-actin, and they observe that terminal ADP-Pi subunits dissociate 5-10 fold more slowly from the barbed ends of F-actin filaments than do ADP subunits, and suggest that the release of Pi is accompanied by a destabilization of actin-actin interactions within the filament allowing subsequent fast dissociation of ADP.

5.6.4 The stability of the actin filament in the presence of bound Pi may be related to the report of Janmey et al (1990) that F-actin filaments assembled with bound ATP are stiffer and straighter than F-ADP actin filaments: the latter appear convoluted and entangled. However, Janmey et al also report that the addition of Pi does not, apparently, cause F-ADP actin filaments to stiffen, and conversely, that F-ATP actin filaments retain their original properties at steady state, after ATP has been hydrolysed. They suggest that this may be because the conformation is trapped in a strained state.

5.6.5 Both these sets of observations suggest that an F-actin filament may, according to conditions, be in any one of two or more states, but that at any one time the

whole filament is in the same state, with all the subunits in the same conformation or state of charge. This accords with the gel observations in this work.

5.6.6 However, De Rosier and Tilney (1984), in a wide-ranging review chapter on the Form & Function of Actin, observe that the angle between adjacent subunits in a single filament may vary, a phenomenon they call angular disorder. De Rosier and Tilney suggest that this property, the flexibility and adaptability of the actin filament, is particularly important for the function of actin: "What is exciting and eminently satisfying to a structural biologist is that the actin filament is designed to accommodate any packing arrangement simply by making use of its angular disorder."

5.6.7 Angular disorder means that although each actin subunit has a fixed axial displacement of 2.73nm relative to its neighbours (ie is the same distance apart along the filament axis), its angular displacement varies by  $\pm 10^\circ$  from  $167^\circ$  (ie a variable pitch of twist of the helix). Egelman & De Rosier (1983) developed a model of the actin filament consisting of bi-lobed subunits capable of variable tilt, and giving an outer filament diameter of about 9.5nm. Their model is remarkably consistent with the model of an actin filament by Holmes et al (1990) based on the determination of the crystal structure of the actin-DNase complex by Kabsch et al (1990): Holmes et al also give a maximum filament diameter of 90-95 Å.

5.6.8 Angular disorder allows flexible actin filaments to cross-link into bundles even when they are in a disordered and entangled state (Kane, 1976). With time (hours) they develop more ordered, regular hexagonal packing. It seems likely that this property is implicated in the differences between stirred and sonicated F-actin gels.

5.6.9 Angular disorder also allows the formation of cross-linked bundles of filaments with bends built into them (De Rosier and Tilney, 1984). It is therefore possible for actin to form hexagonally packed, cross-linked, curved, bent or wavy structures which are equally as stable as arrays of straight filaments.

5.6.10 The phenomenon of actin treadmilling has been studied by many researchers, including Wegner (1976), Pollard and Mooseker (1981) and Coluccio and Tilney (1983). Treadmilling is a factor of the polarity of the actin filament, and is the phenomenon of preferential net addition of monomers at one end of the filament and net disassembly from the other end. When the polarity is identified by the decoration of F-actin filaments with the S-1 fragment of myosin, the barbed end is the preferred end for addition, and the pointed end for disassembly.

5.6.11 In principle this must mean not only that the actin filament can move in the direction of the barbed end, but also that it does so in a screw motion as new subunits attach in a helical pattern. This might have important implications for the function of actin and for its ability to reassemble or to re-align its cross-links after different types of disruption. In practice, however, it appears that under physiological conditions this process takes place too slowly to be significant (Coluccio and Tilney, 1983). In the intact cell it is possible that the process is in some way accelerated by accessory proteins, but this has not so far been observed.

5.6.12 The atomic structure of actin has recently been determined by Kabsch et al. (1990) from the structure of the actin : DNase 1 complex. The actin molecule has two domains, each divided into two subdomains, with the nucleotide bound in the cleft between the two domains. The S-1 binding site appears to be in the small domain, with residues 1-113 forming a looped subdomain and a cleft between this

and the neighbouring subdomain, into which S-1 is likely to fit. Alternatively, in an earlier paper Holmes et al (1990a) suggest that S-1 is in contact with the small domains of two different subunits, but their more recent model makes this seem less likely.

5.6.13 Holmes et al (1990b) have extended this structure to model the actin filament, fitting together the models of actin subunits into a helical framework. In their model the large domain fits along the actin helix, with binding sites to three adjacent subunits mainly also in the large domain, and also on the looped subdomain formed by residues 40-50 in the small domain. It is in the small domain that most of the residues previously identified as involved in actin polymerization are situated.

Modification of His 40, at the start of the loop, inhibits polymerization, and also inhibits the activation of myosin ATPase (Hegyi et al, 1974). His 40 is within the area identified with S-1 binding but is not within the cleft between the two subdomains, so it may have an indirect function in S-1 binding, such as affecting the conformation of the cleft. In the F-actin state, once actin has polymerized, His 40 is not susceptible to chemical modification, suggesting that it is protected by its proximity to, or interaction with, a neighbouring subunit, although the model by Holmes et al suggests that this must be a long-range interaction.

5.6.14 Bender et al (1976) found that reacting Tyr 53 with 5-diazonium-(1H)[<sup>14</sup>C] tetrazole in G-actin completely prevented actin polymerization; again, this residue is not accessible to the reagent in F-actin. Tyr 53 is involved in the site of S1 binding but is not close to any of the suggested sites of actin subunit interaction. Holmes et al suggest that its role is in the stabilization of the 40-50 loop.

5.6.15 Similarly, nitration of Tyr 69 is also found to prevent polymerization (Schutt and Lindberg, 1990) or at least to impair it, although it is in the cleft between the two subunit domains rather than directly involved in binding between subunits. All these findings suggest that there are still important questions to be answered about the interactions within the actin filament.

5.6.16 Holmes et al (1990a) have also added tropomyosin to their model of F-actin structure. In this model tropomyosin makes contact only with the large domain, the domain closer to the filament axis. This position, in the groove between the two actin helices, accords well with the classic thin filament model (Moore et al 1970).

5.6.17 Egelman and De Rosier (1983 b) argue that although there is no cumulative axial displacement of actin subunits, there is evidence for some tilting of subunits, giving the effect of local axial displacement. Subunits are found to be at variable angles to the axis, ranging from 20-30 degrees to an almost transverse position. This tilting subunit hypothesis accounts for the forbidden meridional reflections in paracrystals observed in a number of studies. Holmes et al (1990a) suggest that movement of the outer (small) domain, causing the tilt, is a response to electrostatic forces which come into play during close packing in concentrated gels; it would seem from this model that there is a flexible hinge between the two domains. (Concentrated gels in this case usually mean gels of 30-50 mg/ml, corresponding to the concentration of stirred or sonicated gels used in this work, rather than to those which have been ultracentrifuged).

5.6.18 All these various findings have added considerably to the understanding of the actin molecule and to the appreciation of its unique range of properties. Its ability to bind or shed ions according to different conditions is wholly consistent with

the emerging picture of an exceedingly flexible and adaptable molecule with a large number of functions.

### **Section 5.7 : Charge on S-1 and myosin rod**

5.7.1 The charge on the S-1 portion of myosin (the myosin head, produced by papain digestion) was derived from the charge on the combined gel of actin-S-1, since S-1 will bind to actin but will not on its own form a gel. The calculation assumes that the charge on actin is not changed by binding S-1 (see Section 5.2.1 - 5.2.2), and that the S-1 charge can be arrived at by subtracting the charge found on the control actin from the charge produced by the combined gel.

5.7.2 The S-1 charge and the charge found on the other portion of the myosin molecule, the myosin rod, can, together, be compared with the charge on the intact myosin molecule (Table 3.19). At the higher ionic strengths, A and 0.75A, the myosin rod charge is a high proportion of the intact myosin charge, and the rod and S-1 charges together approximate to the charge in the myosin gel, as might be predicted. There is, however, an increasing discrepancy as ionic strength is lowered. The S-1 charge decreases to zero in 0.5A and becomes positive in 0.2A; the rod charge also falls more sharply than the whole myosin charge (Fig 3.2), such that in 0.2A the net result of the combination of the rod and S-1 charges is only 9 e/molecule, in contrast to the whole myosin charge of 48 e/molecule.

5.7.3 This suggests that the two parts of the molecule are more affected by changes in ionic strength when they are separate than when they are together, and that the whole myosin molecule achieves some sort of cooperative buffering. It is possible, alternatively, that the binding of S-1 to actin (without which it will not aggregate into



a gel) affects either the S-1 or the actin itself, or indeed both, rendering them more susceptible to ionic strength changes, and therefore that this corresponds in some way to the rigor state in intact fibres. However, this is in conflict with charge findings in intact fibres, where the charge is considerably higher in rigor than in relaxation.

5.7.4 The S-1 charge is calculated on the assumption that the charge on the actin with which it is complexed is not affected by it. The S-1 charge, ranging from 9e in A solution to 3+ in 0.2A, is within the range predicted by sequence data of between 17e and 7+, depending on histidine ionization. However, it is interesting that there appears to be no significant change in the actin charge to reflect the increased order which Egelman (1985) reports is imposed by S-1 binding (5.4.10). Since there is no independent measure of the charge of either protein when they are complexed, the S-1 charge calculation is dependent on the control actin. If the actin charge had been raised by the S-1 binding, the corollary would be that the S-1 must have a lower charge, and vice versa. Either of these situations is, of course, possible, and in vivo the release of Pi after ATP splitting could well make the myosin head more positive and the actin molecules of the thin filament more negative, in a cyclic fashion. There is no evidence, though, that charge changes are occurring to the proteins in the gel. The charge found in gels of actomyosin (section 5.8) gives a similar result.

5.7.5 The myosin rod charge (Table 3.3) is lower than would be predicted by sequence data, from which a maximum of 94e would be expected, falling to 60e if all histidines were ionized. If the charge recorded in the gel is accurate, myosin rod may be binding positive ions (potassium is a potential candidate). Perhaps in the

intact myosin molecule S-1 has a stabilising role which isolated myosin rod then seeks to supply by ion-binding.

### **Section 5.8 : Charge characteristics of actomyosin gels**

5.8.1 The results of charge measurements of gels of a combination of actin and myosin, actomyosin, appear to show that the two proteins behave in combination in the same way as they do separately: that is, combining the two does not seem to produce a significant change in either (unless equal and opposite changes are produced leading to the same net combined charge: this seems unlikely).

5.8.2 The process of measuring the actomyosin charge did, however, highlight two unexpected and unexplained features of this gel, see section 3.3.3. These were the two discrete peaks of readings observed, and the creep effect from one level of reading to a second.

5.8.3 The first possible explanation for the double-peak effect is that the two proteins, actin and myosin, were imperfectly mixed and that instead of measuring an actomyosin charge the microelectrode was reading charges from each of the two separate proteins. Insofar as it is possible to calculate the hypothetical charges of the two proteins from the two peaks, the lower of the two might be the myosin and the higher peak the actin. This would correspond with actin's higher charge per unit volume.

5.8.4 A number of considerations militate against this explanation, however. First, it seems improbable in view of the thoroughness of the mixing, although any judgement as to thoroughness is necessarily subjective. The mixture was repeatedly

syringed through very fine-bore piping after exhaustive hand-stirring, and it is hard to see how any but a few molecules could have remained apart from each other. This effect was not seen in any other of the various protein combinations, all of which were mixed in the same way. Second, this explanation suggests that none of the protein was mixed, the entire gel being in the form of alternative domains. This also seems almost impossible (and analogous to Maxwell's Demon), whereas a histogram in the form of a bell curve with a very wide base might more plausibly have suggested that the majority of the protein was mixed but that small percentages of each remained separate. Third, the proportions of the two proteins in the gel were different, with an approximate ratio of myosin : actin:: 3 : 1 by volume: this would be expected to be reflected in the histogram, whereas the number of readings in each peak is almost equal. Fourth, the electron micrograph of actomyosin shows no discrete sections of either protein. Finally, the hypothesis which invokes separated proteins does not account for the second feature of actomyosin gels, the creep effect. It may be that the two features are entirely unrelated, but as neither occurs in any other gel it is more likely that they represent two aspects of the same phenomenon.

5.8.5 Another possibility is that the two peaks show actomyosin in two different states, and that the creep effect shows it changing from one state to the other. On this hypothesis one state is more highly charged than the other; this could perhaps be the result of two different types of interaction between the actin and myosin molecules. It is possible to speculate that this might be analogous, if not identical, to the cycle of interactions between the A-band and the I-band in the intact fibre. However, in this case the cycle is not being propelled by the availability of ATP; and in the absence of tropomyosin-troponin there would seem to be no reason for myosin not to attach to actin and remain there.

5.8.6 It is perhaps conceivable that the insertion of the microelectrode exerts some mechanical force which produces a change in the conformation or binding state of the molecules in its vicinity. This might be similar to stirring the actin gel. However, it is hard to envisage this occurring with half the insertions and not with the other half. Again, too, such an effect was not observed with any other gels.

5.8.7 It is puzzling that these effects were apparent in some of the actomyosin gels measured but not in all of them. It may be that the effects were there but were masked by a generally wide spread of readings, or that in some cases the two peaks overlapped, but an examination of the data gives no suggestion that this was the case. There were no discernable differences between preparations or procedures which might account for differences in characteristics.

5.8.8 What remains, therefore, is an intriguing phenomenon into which further investigation is needed. It is another indication that the interactions between these proteins are not straightforward, and are governed by factors not yet identified.

## CONCLUSION

This study of the charge characteristics of muscle proteins was designed to complement the work being done on the charge found in intact muscle fibres (Bartels & Elliott 1981, 1985), and thus, it was hoped, to illuminate the roles played by different proteins in the changes in charges which had already been observed. The intention was to develop the techniques of microelectrode measurement and to identify their role in further experimentation.

The study has answered some questions, in particular confirming that myosin and actin are the major contributors to the charge found in, respectively, the A- and I-bands of intact muscle fibres. It has also raised new questions and highlighted areas for further study. The correspondence between the charge found in protein gels and that in intact muscle, and in particular the discrepancy between myosin and A-band charge, require continued investigation, with further work on the contribution of C-protein and nucleotide.

An intriguing question concerns the curious property of the low-concentration F-actin gel: what is happening to produce a charge increase when the gel is stirred? The recent developments in modelling the structure of actin make this question particularly apposite, as it is now possible to predict with some confidence the positions of the charged residues. Further work would examine the low-concentration actin phenomenon under different experimental conditions, building on the observations of the high-concentration gels of F-actin in combination with other proteins, in particular with myosin and with tropomyosin-troponin.

Actin and myosin are key proteins, and the elucidation of their characteristics is central to the growing understanding of the function of muscle systems. The factors affecting their charge should provide vital pieces towards the completion of the jigsaw.

## APPENDIX: ELECTRICAL DOUBLE LAYERS IN BIOLOGICAL SYSTEMS

The concept of the electrical double-layer was first described by Gouy, Chapman, and Debye & Huckel; this early work was reviewed by Verwey & Overbeek (1948).

Biological macromolecules include in their structure a number of amino acids with ionizable side groups (acidic groups are aspartate, pK 3.86 and glutamate, pK 4.25; basic groups are lysine, pK 10.53, arginine pK 12.48 and histidine, pK 6.5). In solution, therefore, these macromolecules are polyelectrolytes, that is, they carry a net electrical charge. In most cases at physiological pH (pH 7.0-7.5) this is a net negative charge. (In muscle myosin, there are approximately 100 positive groups and 110 negative groups for every  $10^5$  daltons).

In these circumstances, as Verwey & Overbeek (1948) say,

"....each particle charge is screened off by an equivalent swarm of (counter-) ions surrounding the particle. Hence, if we consider one particle separately, immersed in the liquid, it is surrounded by an electric double-layer. One layer is formed by the charge in the surface of the particles. Though in reality it is a charge consisting of point charges, it is customary to consider it, as a first approximation, as a homogeneous surface charge spread over the surface of the particles.

The second layer is formed by the oppositely-charged ions in the solution..."

In a network of charged macromolecules and in the absence of physical links, stability is maintained by the balance between long-range attractive forces and electrostatic repulsive forces. The former are the London-van der Waals attractions, which result from "the polarization of one atom by the fluctuations of

the charge distribution in a second atom, and vice versa" (Verwey & Overbeek 1948) - in other words from a rapidly-fluctuating dipole moment. These attractions are additive, in that each atom attracts all other atoms; thus the attractions are additive, in that each atom attracts all other atoms; thus the attractive force between two large macromolecules will be significant.

The opposite, repulsive force arises simply because similarly-charged molecules repel each other, through the interaction of their oppositely orientated electrical double-layers. The extent of this repulsive force is dependent on two factors, the level of charge on the molecules and the ionic strength of the surrounding medium.

The level of charge on each molecule is primarily dependent on pH: a high pH gives rise to a higher negative charge as more side groups become ionized. (The isoelectric point of any polyelectrolyte is the pH at which the molecule is electrically neutral as the negative and positive charges are balanced).

As the ionic strength of the medium is increased, however, the increasing number of ions in solution act as a barrier to the operation of the repulsive force, reducing its effectiveness. This is the screening effect, which was demonstrated in studies of tobacco mosaic virus (TMV) by Bernal & Fankuchen (1941); in the present study it is shown in the effect of ionic strength on the concentration of ultracentrifuged actin (Fig 3.3).

The net surface charge on a macromolecule in solution may also change as a function of ionic strength, due to ion binding (or ion association). This effect is shown to be important in the gels used in this study.



Rome (1967) showed, in X-ray diffraction studies, that the interfilament spacing in glycerol-extracted muscle filaments varied as a function of pH and ionic strength. She inferred that there must be electrical repulsive forces between the filaments. She also showed (1968) that the filament lattice could be compressed by increasing the osmotic pressure outside a fibre with an intact membrane. Millman & Nickel (1980) took this further, osmotically compressing muscle lattices without intact membranes by the use of large polymers which did not penetrate the filament lattice; they did similar experiments with gels of TMV. Millman & Nickel explained their results using electrical double-layer theory, solving the Poisson-Boltzmann equation (Verwey & Overbeek 1948) in cylindrical co-ordinates.

In muscle, as opposed to TMV and to the types of gel considered by Verwey & Overbeek, there are certainly physical links between filaments, as well as London-Van der Waals attractive forces. In intact filaments these may take the form of protein links (eg M-line, Z-band). In the case of the gels used in this study the molecules may be entangled together, since the spinning-down process produces a disordered gel. The cohesion produced by these physical links is likely to be of an order of magnitude greater than the London-Van der Waals forces, which in these circumstances are insignificant by comparison.

In muscle, at physiological pH, there are about  $4 \times 10^4$  negative charges (electrons) per filament. Each filament is in the order of  $2 \mu\text{m}$  in length, giving one charge for approximately every 0.2nm. This gives rise to a surface potential of several tens of millivolts, similar to that of a membrane potential. Shortly after Rome's work on muscle filaments, Collins & Edwards (1971) developed their method of measuring Donnan potentials using microelectrodes. This allowed Rome's work to be advanced by Elliott and Naylor (see Naylor 1977) and Bartels & Elliott (1981), who

made direct measurements of charge on glycerol-extracted or chemically skinned muscle fibres. More recently, Bartels & Elliott (1985) developed a mechanism of accurate microelectrode impalement of the A- and I-bands of muscle fibres, separately, allowing direct measurement of the charge on each.

The present study now extends this for the first time to the direct measurement of charge on the main protein components of the A- and I-bands, myosin and actin respectively.

## Bibliography

R. H. Adrian 1956: The effect of internal and external potassium concentrations on the membrane potential of frog muscle. *J. Physiol. (Lond.)* 133: 631-658.

S. Asakura, 1961: F-actin adenosine triphosphatase activated under sonic vibration. *Biochim. Biophys. Acta*, 52: 65-75.

S Asakura, M. Taniguchi & F. Oosawa 1963: Mechano-chemical behaviour of F-actin. *J. Mol. Biol.* 7: 55-69.

E. M. Bartels & G. F. Elliott 1981: Donnan potentials from the A- and I-bands of skeletal muscle, relaxed and in rigor. *J. Physiol. (Lond.)* 317: 85-87P.

E. M. Bartels & G. F. Elliott 1985: Donnan potentials from the A- and I-bands of glycerinated and chemically skinned muscles, relaxed and in rigor. *Biophys. J.* 48: 61-76.

E. M. Bartels, P. H. Cooke, G. F. Elliott, R. A. Hughes [in preparation]: The myosin molecule - charge response to nucleotide binding. Forthcoming.

N. Bender, H. Fasold, A. Kenmoku, G. Middelhoff & K. E. Volk 1976: The selective blocking of the polymerization reaction of striated muscle actin leading to a derivative suitable for crystallization. *Eur. J. Biochem.* 64: 215-218.

J. D. Bernal & I. Fankuchen 1941: X-ray and crystallographic studies of plant virus preparations. *J. Gen. Physiol.* 25: 111-146.

M-F. Carlier & D. Pantaloni 1988: Binding of phosphate to F-ADP- actin and role of F-ADP-Pi-actin in ATP-actin polymerization. *J. Biol. Chem.* 263: 817-825.

E. W. Collins & C. Edwards 1971: Role of Donnan equilibrium in the resting potentials in glycerol-extracted muscle. *Am. J. Physiol.* 221: 1130-1133.

J. H. Collins & M. Elzinga 1975: The primary structure of actin from rabbit skeletal muscle. *J. Biol. Chem.* 250: 5915-5920.

J. H. Collins, M. L. Greaser, J. D. Potter, M. J. Horn 1977: Determination of the amino acid sequence of troponin C from rabbit skeletal muscle. *J. Biol. Chem.* 252: 6356-6362.

L. M. Coluccio & L. G. Tilney 1983, - Under physiological conditions actin disassembles slowly from the nonpreferred end of an actin filament. *J. Cell. Biol.* 97: 1629-1634.

P. H. Cooke, E. M. Bartels, G. F. Elliott & R. A. Hughes 1987: A structural study of gels, in the form of threads, of myosin and myosin rod. *Biophys. J.* 51: 947-957.

D. J. De Rosier and L. G. Tilney 1984: The form and function of actin in *Cell and Muscle Motility* vol 5 *The Cytoskeleton*, ed. J. W. Shay, Plenum Pub. Corp. (NY).

C. M. P. Deshayes, E.. M. Bartels, G. Ojteg & G. F. Elliott 1991: Charge measurements on I-filament proteins. *J. Muscle Res. Cell Motility* 12 : 113.

S. Ebashi 1972: Calcium ions and muscle contraction. *Nature* 240: 217-218.

S. Ebashi, M. Endo and I. Ohtsuki 1969: A model for the fine structure of the thin filament. *Quart. Rev. Biophys.* 2: 466.

E. H. Egelman 1985: The structure of F-actin. *J. Muscle Res. Cell Motility* 6: 129-151.

E. H. Egelman and D. J. De Rosier 1983a: A model for F-actin derived from image analysis of isolated filaments. *J. Mol. Biol.* 166: 623-629.

E. H. Egelman and D. J. De Rosier 1983b: Structural studies of F-actin. In *Actin: Structure and Function in Muscle and Non-Muscle Cells*, ed. C. G. dos Remedios & J. A. Barden, Academic Press, pp 17-24.

G. F. Elliott 1973: Donnan and osmotic effects in muscle fibres without membranes. *J. Mechanochem. Cell Motility* 2: 83-89.

G. F. Elliott 1992: the contractile process. Abstracts of European Muscle Club XX Annual Meeting, *Journal of Muscle Research*: In press.

G. F. Elliott & E. M. Bartels 1982: Donnan potential measurements in extended hexagonal polyelectrolyte gels such as muscle. *Biophys. J.* 38: 195-199.

G. F. Elliott, E. M. Bartels and R. A. Hughes 1985: The myosin filament: charge amplification and charge condensation. In *Electrical Double Layers in Biology*, ed. Martin Blank, Plenum Publishing Corp., NY, pp 277-285.

R. J. A. Grand 1982: The structure and function of myosin light chains. *Life Chem. Rep.* 1: 105-160.

L. M. Griffith & T. D. Pollard 1982: Cross-linking of actin filament networks by self-association and actin-binding macromolecules. *J. Biol. Chem.* 257: 9135-9142.

J. Hanson & J. Lowy 1963: The structure of F-actin and of actin filaments isolated from muscle. *J. Mol. Biol.* 6: 46-60.

W. F. Harrington 1979: The contractile proteins of muscle. In *The Proteins IV* (3rd edn.), ed. H. Neurath & R. L. Hill, Academic Press.

G. Hegyi, G. Premecz, B. Sain, A. Muhrad 1974: Selective carbethoxylation of the histidine residues of actin by diethylpyrocarbonate. *Eur. J. Biochem.* 44: 7-12.

K. C. Holmes, D. Popp, W. Gebhard & W. Kabsch 1990a: The structure of F-actin, thin filaments, and decorated thin filaments. *J. Mol. Biol.* 50: 279-295.

K. C. Holmes, D. Popp, W. Gebhard & W. Kabsch 1990b: Atomic model of the actin filament. *Nature* 347: 44-49.

A. F. Huxley & R. Niedergerke 1954: Interference microscopy of living muscle fibres. *Nature* 173: 971-973.

H. E. Huxley & J. Hanson 1954: Changes in the cross-striations of muscle during contraction and stretch and their structural implications. *Nature* 173: 973-976.

P. A. Janmey, S. Hvidt, G. F. Oster, J. Lamb, T. P. Stossel, J. H. Hartwig 1990: Effect of ATP on actin filament stiffness. *Letters to Nature, Nature* 347: 95-99.

K. Jennison, G. F. Elliott and C. Moos 1981: Charge measurements of muscle proteins. *Biophys. J.* 33: 26a.

W. Kabsch, H. G. Mannherz, D. Suck, E. F. Pai, K. C. Holmes 1990: Atomic structure of the actin-DNAse I Complex. *Nature* 347: 37-44.

R. E. Kane 1976: Actin polymerization and interaction with other proteins in temperature-induced gelation of sea urchin egg extracts. *J. Cell. Biol.* 71: 704-714.

J. Karn, S. Brenner & L. Barnett 1983: Protein structural domains in the *caenorhabditis elegans* unc-54 myosin heavy gene chain are not separated by introns. *Proc. Nat. Acad. Sci. USA* 80: 4233-4257.

R. W. Kensler & M. Stewart 1983: Frog skeletal muscle thick filaments are three-stranded. *J. Cell Biol.* 96: 1797-1802.

J. Koretz 1982: Hybridization & Reconstitution of Thick-Filament Structure. *Methods in Enzymology* 85: 20-31.

E. D. Korn, M-F. Carrier, D. Pantaloni 1987: Actin polymerization and ATP hydrolysis. *Science*, 238: 638-644.

G. I. Loeb & H. A. Saroff 1964: Chloride- and hydrogen-ion-binding to ribonuclease. *Biochemistry* 3: 1819.

R. C. Lu & M. Elzinga 1977: Partial amino-acid sequence of brain actin and its homology with muscle actin. *Biochemistry* 16: 5801-5806.

A. D. McLachlan & J. Karn 1982: Periodic charge distributions in the myosin rod amino acid sequence match cross-bridge spacing in muscle. *Nature* 299: 226-231.

B. M. Millman & B. G. Nickel 1980: Electrostatic forces in muscle and cylindrical gel systems. *Biophys. J.* 32: 49-63.

P. B. Moore, H. E. Huxley & D. J. De Rosier 1970: Three dimensional reconstruction of F-actin, thin filaments, and decorated thin filaments. *J. Mol. Biol.* 50: 279-295.

C. Moos, G. Offer, R. Starr, P. Bennett 1975: Interaction of C-protein with myosin, myosin rod and light meromyosin. *J. Mol. Biol.* 97: 1-9.

C. Moos & I. N. M. Feng 1980: Effect of C-protein on actomyosin ATPase. *Biochim. & Biophys. Acta* 632: 141-149.

G. R. S. Naylor 1977: X-ray and microelectrode studies of glycerinated rabbit psoas muscle. PhD thesis, The Open University.

G. R. S. Naylor 1978: A simple circuit for automatic continuous recording of microelectrode resistance. *Pfluegers Arch. Eur. J. Physiol.* 378: 107-110.



G. R. S. Naylor, E. M. Bartels, T. D. Bridgman, G. F. Elliott 1985: Donnan potentials in rabbit psoas muscle in rigor. *Biophys. J.* 48: 47-59.

G. Offer, C. Moos & R. Starr 1973: A new protein of the thick filaments of vertebrate skeletal myofibrils. *J. Mol. Biol.* 74: 653-676.

F. Oosawa, S. Asakura & T. Ooi 1961: Physical chemistry of muscle protein actin. *Progress of Theoretical Physics supplement* 17: 14-34.

J. R. Pearlstone, P. Johnson, M. R. Carpenter, L. R. Smillie 1977: Primary structure of rabbit skeletal muscle troponin T. *J. Biol. Chem.* 252: 983-989.

D. D. Perrin & I. G. Sayce 1967: Computer calculation of equilibrium concentrations in mixtures of metal ions and complexing species. *Talanta* 14: 833-842.

T. D. Pollard & M. S. Mooseker 1981: Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell Biol.* 88: 654-659.

E. S. Reynolds 1963: The Use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17: 208

E. Rome 1967: Light and X-ray diffraction studies of the filament lattice of glycerol-extracted muscle. *J. Mol. Biol.* 27: 591-602.

- E. Rome 1968: X-ray diffraction studies of the filament lattice of striated muscle in various bathing media. *J. Mol. Biol.* 37: 331-344.
- C. E. Schutt & U. Lindberg 1990: The nature of the actin molecule. In *Molecular Mechanisms in Muscle Contraction*, ed. J. M. Squire, Macmillan, p49-63.
- L. G. Sillen and A. E. Martell 1964, 1971: Stability constants Supplement No 1, Chemical Society Special Publications nos 17 (1964) and 25 (1971).
- H. S. Slayter & S. Lowey 1967: Substructure of the myosin molecule as visualised by electron microscopy. *Proc. Nat. Acad. Sci. USA* 58: 1611-1618.
- D. Stone & L. B. Smillie 1978: The amino-acid sequence of rabbit skeletal tropomyosin. *J. Biol. Chem.* 253: 1137-1148.
- S. J. Tumminia, J. F. Koretz & J. C. Landau 1989: Myosin aggregates at pH 7.0 with and without C-protein. *Biochim. Biophys. Acta* 999: 300-312.
- J. Vanderkerckhove & K. Weber 1978: Comparison of three mammalian cytoplasmic actins. *Eur. J. Biochem.* 90: 451-462.
- E. J. W. Verwey & J. Th. G. Overbeek 1948: Theory of the stability of Lyophobic Colloids. Elsevier Publishing Company, Amsterdam-New York.
- F. Wang, R. V. Sampogna & B. R. Ware 1989: pH dependence of actin self-assembly. *Biophys. J.* 55: 293-298.

A. Wegner 1976: Head to tail polymerization of actin. *J. Mol. Biol.* 108: 139-150.

J. M. Wilkinson & R. J. A. Grand 1975: Amino acid sequence of troponin I from rabbit skeletal muscle. *Biochem. J.* 149: 403-496.

K. Yamamoto & C Moos 1983: The C-proteins of rabbit red, white and cardiac muscles. *J. Biol. Chem.* 258: 8395-8401.